THE UNIVERSITY of ADELAIDE

Peripheral-to-Central Neuroimmune Communication and the Sun: Implications for Addiction and Neurodegenerative Disease Pathology

A thesis submitted in fulfilment for the degree of

MASTER OF PHILOSOPHY

in

The Discipline of Physiology

Adelaide Medical School

The University of Adelaide

by

Krystal Lee Iacopetta

March 2019

Table of Contents

Thesis declaration ..... **ix**

Acknowledgments ..... **x**

Thesis abstract and outline ..... **xi**

Abbreviations ..... **xiii**

Chapter 1. General introduction: Ultraviolet radiation and the brain **1**

1.1 Solar electromagnetic radiation has important implications for life on earth 1

1.1.1 Ambient atmosphere influences the amount of ultraviolet radiation in the immediate environment 3

1.1.2 Electromagnetic energy of solar radiation is biologically active 4

1.2 Cutaneous responses to ultraviolet radiation exposure 6

1.2.1 The structure and function of human skin 6

1.2.2 Negative consequences of ultraviolet radiation exposure 8

1.2.3 Health benefits of ultraviolet radiation exposure 9

1.2.4 New challenges of ultraviolet radiation related to skin pigmentation in the modern world 11

1.3 Skin-brain axis: How ultraviolet radiation "touches" the brain 12

1.4 The innate immune system facilitates peripheral-to-central communication 15

Chapter 2. Can neuroimmune mechanisms explain the link between ultraviolet light exposure and addictive behaviour? **21**

2.1 Abstract 22

2.2 Introduction 22

2.3 Evidence to suggest that tanning is comparable to behavioural addictions 25

2.4 Why the immune system is relevant to addiction 28
2.5 Ultraviolet radiation exposure damages skin cells contributing to release of endogenous danger signals ("alarmins") that activate the immune system 30
2.6 Alarmins contribute to inflammation through binding to pattern recognition receptors such as TLR4, leading to downstream release of pro-inflammatory products.....34
2.7 Peripheral TLR4 activation can influence neuronal signalling and alter behaviour through immune-brain communication pathways 36
2.8 We don't like being sick, so why do we "like" UV exposure?.....40
2.9 Conclusion 43
Thesis aims and hypotheses 45
Chapter 3. Do UV-induced alarmin stress signals influence dopaminergic brain pathways and increase motivation?.....47
3.1 Brief summary.....47
3.2 Research questions 48
3.2.1 Is UVR associated with increased motivational output and the continued engagement in operant lever pressing? Does this correlate with elevated production of alarmins and immune activation? 48
3.2.2 Is UV exposure associated with increased dopamine within the nucleus accumbens in a dose dependent manner? 50
3.3 Aims 51
3.4 Hypotheses.....52
3.5 Experiment 1: Examine the effect of daily ultraviolet exposure on motivational drive and determine ultraviolet induced alterations in the alarmin response in rodents.....52
3.6 Experiment 2: Determine the effect of ultraviolet exposure on the levels of dopamine within the nucleus accumbens 53

3.7 Proposed methodology 54
3.7.1 Animals 55
3.7.2 Ultraviolet light apparatus 55
3.7.3 Behavioural testing 57
3.7.4 Tissue collection 58
3.7.5 Histological analysis 59
3.7.6 Immunohistochemistry 60
3.7.7 Molecular pathology 61
3.7.7.1 Tissue processing 61
3.7.7.2 Western blot 61
3.7.7.3 Enzyme-linked immunosorbent assay (ELISA) 62
3.7.8 Microdialysis and high-performance protein liquid chromatography (HPLC) 63
3.8 Significance 64
Chapter 4. Are the protective benefits of vitamin D in neurodegenerative disease dependent on route of administration? A systematic review 69
4.1 Abstract 70
4.2 Background 72
4.2.1 Possible mechanisms by which vitamin D may contribute protective benefit within neurodegenerative disease 74
4.2.2 Gaps in current evidence surrounding therapeutic benefit of vitamin D 76
4.3 Methods 77
4.3.1 Corpus data analysis and visualisation 77
4.3.2 Search strategy for systematic review 78
4.3.3 Systematic review publication selection criteria 79
4.3.4 Systematic review publication data extraction 79

4.4 Results 81
4.4.1 Corpus data analysis summary 81
4.4.1.1 Description of the vitamin D field of research 81
4.4.2 Systematic review search outcomes 84
4.4.3 Multiple sclerosis 86
4.4.3.1 Pre-clinical studies 86
4.4.3.2 Case-control studies 88
4.4.3.3 Cross-sectional studies 90
4.4.3.4 Prospective studies 91
4.4.4 Parkinson's disease 94
4.4.4.1 Pre-clinical studies 94
4.4.4.2 Case-control studies 95
4.4.4.3 Cross-sectional studies 96
4.4.4.4 Prospective study 96
4.4.5 Alzheimer's disease and dementia 97
4.4.5.1 Pre-clinical studies 98
4.4.5.2 Longitudinal cohort studies 99
4.4.5.3 Prospective studies 100
4.4.6 Amyotrophic lateral sclerosis 102
4.4.6.1 Pre-clinical studies 102
4.4.6.2 Longitudinal cohort study 103
4.5 Discussion 119

Chapter 5. Discussion 127

5.1 Summary of thesis outcomes 127
Chapter 2. A link between ultraviolet radiation and addiction 127

Chapter 3. Does UV-induced alarmin signalling influence dopaminergic transmission and increase motivation? 128

Chapter 4. Does source of vitamin D influence neurological effects? 128

5.2 General discussion and future directions 129

5.3 Concluding remarks 134

References 135

Appendices 159

Appendix A: Design of novel apparatus for the delivery of targeted ultraviolet radiation on the skin 161

Appendix B: Pilot results 165

Pilot 1A: Acute ultraviolet exposure to assess skin response 165

Pilot 1B: Repeated ultraviolet exposure to assess skin response after 7 days 172

Appendix C: Source code written for operant conditioning boxes 178

Magazine training 179

Fixed ratio reinforcement schedule 180

Progressive ratio reinforcement schedule 183

Thesis declaration

‘I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or tertiary institution and, to the best of my knowledge and belief contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in future, be used in a submission in my name, for any other degree or diploma in any university of tertiary institution without prior approval of the University of Adelaide and where applicable, any partner institutions responsible for the joint-award of this degree.’

‘I acknowledge that copyright of published works contained within this thesis resides with the copyright holder(s) of those works.’

‘I also give permission for the digital version of my thesis to be made available on the web, via the University’s digital research repository, the Library Search and also through search engines, unless permission has been granted by the University to restrict access for a period of time’.

‘I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship’.

Krystal Lee Iacopetta

March 2019

Acknowledgments

I extend my thanks and gratitude to those who supported me throughout this journey. In particular to my supervisors, Professor Mark Hutchinson for your endless positivity and excitement, Dr. Femke Buisman-Pijlman for your thoughtful considerations and Dr. Lyndsey Collins-Praino for your valuable input and advice.

Thank you to the members of the Neuroimmunopharmacology Laboratory for creating an exceptional work family, filled with friendship, laughter and continual support.

Lastly, thank you to my family, for always being there and giving exactly what I needed.

Thesis abstract and outline

The format of this thesis is as follows: a general introduction, a literature review, a research proposal, a systematic review, a general discussion, references and appendices. Both the literature review and systematic review (Chapters 2 and 4) have been published in peer-reviewed journals and are presented in their **original** manuscript format, except that language had been adjusted into Australian English for consistency, and literature citations have been collated within the reference section.

The work presented herein explores how peripheral ultraviolet light applied to the skin can affect the central nervous system and behaviour, with a focus on the clinical translation to prevalent neurological disorders. First, the impact of solar irradiation on the integumentary system and evidence of skin-brain-communication pathways are introduced. This discussion builds the concept that peripheral UV signals arising in the skin may result in global manifestations via the brain (Chapter 1). The idea of skin-brain communication is further explored with a literature review linking sunbathing, UV exposure seeking and addictive behaviour (Chapter 2). In this chapter, a novel hypothesis is presented suggesting that UV-induced inflammatory signalling may influence neuronal circuits to increase the addictive-like behaviours observed in frequent tanners (Chapter 2). This idea provides the basis for a research proposal (Chapter 3) detailing planned experimental work to investigate whether UV radiation influences mesolimbic dopaminergic systems within the brain, and if inflammation plays a substantial role. Chapter 3 is presented as a research proposal as the work could not be completed due to unforeseen circumstances, which significantly reduced my capacity to continue with the study. Appendices have been included to exhibit the pilot research that had commenced. The final research chapter (Chapter 4) focuses on the role of sun-induced or administered vitamin D and its influence on neurological health. This chapter presents a systematic review of published literature that investigates whether the presumed protective benefits from vitamin D, in neurodegenerative disease, is dependent on route of administration.

Abbreviations

6-OHDA	6-hydroxydopamine
7-DHC	7-dehydrocholesterol
α-MHS	α-melanocyte stimulating hormone
Aβ	Amyloid beta
ACTH	Adrenocorticotrophic hormone
aCSF	Artificial cerebrospinal fluid
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
ALSFRS-R	Amyotrophic lateral sclerosis functional rating scale
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BACE	β-site APP-cleaving enzyme
BBB	Blood brain barrier
BMI	Body mass index
BOW	Bag of words
CAGE	Cut down, annoyed, guilty, eye-opener (questionnaire)
CD	Cluster of differentiation
CFC	Chlorofluorocarbon
CNS	Central nervous system

CRH	Corticotrophin releasing hormone
CVO	Circular ventricular organs
DA	Dopamine
DAMP	Damage associated molecular patterns
DBP	Vitamin D binding protein
DSM-IV-TR	Diagnostic and Statistical Manual of Mental Disorders
EAE	Experimental autoimmune encephalomyelitis
EDSS	Expanded disease severity scale
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FR	Fixed ratio
G-CSF	Granulocyte-colony stimulating factor
GABA	γ-aminobutyric acid
GDNF	Glial cell derived nerve growth factor
H&E	Hematoxylin and eosin
H&Y Scale	Hoehn and Yahr scale
HaCaT	Human keratinocyte cell line
HMGB1	High mobility group box 1
HPA	Hypothalamic-pituitary-adrenal
HPLC	High-performance protein liquid chromatography
HSP	Heat shock protein

ICAM	Intracellular adhesion molecule
ICV	Intracerebroventricular
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP	Intraperitoneal
LPC	Lysophosphatidyl choline
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
MCP-1	Monocyte chemoattractant protein-1
MIP	Macrophage inflammatory protein
MMSE	Mini mental state examination
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyidine
MS	Multiple sclerosis
NAc	Nucleus accumbens
NAD	Non-Alzheimer's dementia
NGF	Nerve growth factor
NHEK	Normal human keratinocyte cell line
NHS	Normal horse serum
NF-κB	Nuclear factor-κ-light-chain-enhancer of active B cells

NLR	NOD-like receptor
NLTK	Natural language toolkit
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
NMDA	*N*-methyl-D-aspartate
NT3	Neurotrophin 3
PD	Parkinson's Disease
PAMP	Pathogen associated molecular patterns
PGE2	Prostaglandin E_2
PRISMA	Preferred reporting items for systematic reviews and meta-analyses
PROG	Progressive ratio schedule
PRR	Pattern recognition receptor
RAGE	Receptor for advanced glycation end products
rCBF	Regional cerebral blood flow
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SITAD	Structured interview for tanning abuse and dependence
SPECT	Single photon emission computed tomography
PBS	Phosphate buffered saline
PD	Parkinson's disease
PDDS	Patient determined disability scale

POMC	Proopiomelanocortin
TBST	Tris-buffered saline with tween 20
TFIDF	Term frequency inverse document frequency
TIR	Toll/interleukin receptor
TLR	Toll-like receptor
Th	T helper
TH	Tyrosine hydroxylase
TNF	Tumour necrosis factor
UPDRS	Unified Parkinson's disease rating scale
UV	Ultraviolet
UVR	Ultraviolet radiation
VCAM	Vascular adhesion molecule
VDM	Vitamin D2-button mushrooms
VDR	Vitamin D receptor
VTA	Ventral tegmental area

Chapter 1. General introduction: Ultraviolet radiation and the brain

This thesis will explore how peripheral ultraviolet radiation (UVR) to the skin can influence central neurocircuitry, and presents a novel hypothesis of skin-to-brain signalling facilitated by the immune system from UV-damaged skin cells. The ubiquitous nature of sunlight means that almost everyone will have some level of UV exposure on a daily basis; which occurs exclusively through the contact with the eyes and skin (Juzeniene et al., 2011; Lucas et al., 2006). Regardless of these two peripheral ports of entry, it has become apparent that the impact of UVR extends beyond the site of contact, affecting the organism as a whole. Historically, this has been attributed to vitamin D production, however, growing evidence suggests other UV-skin related mechanisms could be at play (Autier et al., 2014; Becklund et al., 2010; Slominski, 2015). Therefore, the purpose of this thesis is to explore current evidence surrounding UV exposure on the skin and the downstream central effects, with emphasis on neurological disorders.

This introductory chapter will provide the relevant background information on UVR and how it interacts with skin cells, as the primary point of contact initiating biological reactivity within the body. This chapter will also introduce the pertinent themes of neuroimmune communication explored throughout Chapters 2 and 3.

1.1 Solar electromagnetic radiation has important implications for life on earth

The sun produces an enormous quantity of electromagnetic energy including cosmic rays, gamma rays, x-rays, UVR, visible radiation and infrared radiation. The electromagnetic radiation produced exists on a continuum that can be visualised as a spectrum, segregated by wavelength (Figure. 1.1). All high-energy radiation, including cosmic, gamma and x-rays, are reflected or absorbed by the Earth's atmosphere.

Consequently, ground level radiation is comprised of infrared (53%), visible (44%) and UVR (3%) (Holick, 2016; Slominski et al., 2018). Although UVR contributes the least percentage, it is the most energetic component of solar radiation at the Earth's surface as there an inverse relationship between wavelength and energy, thus lower wavelengths are higher in energy (Figure 1.1). UVR energy is highly biologically active, making it a key determinant of life on Earth, working as both an initiator and driver of evolution (Rapf and Vaida, 2016; Raven et al., 2008). Simple organic molecules harness the energy from UVR, converting photons of light into high-energy chemical bonds, generating molecular complexity. In this way, the energy of UVR is comparable to that of a covalent bond (Slominski et al., 2018; Rapf and Vaida, 2016). Despite the immense changes to Earth's atmosphere in the 3.7 billion years since life began, UVR continues to reach the Earth's surface and affect living tissues (Juzeniene et al., 2011; Lucas et al., 2006).

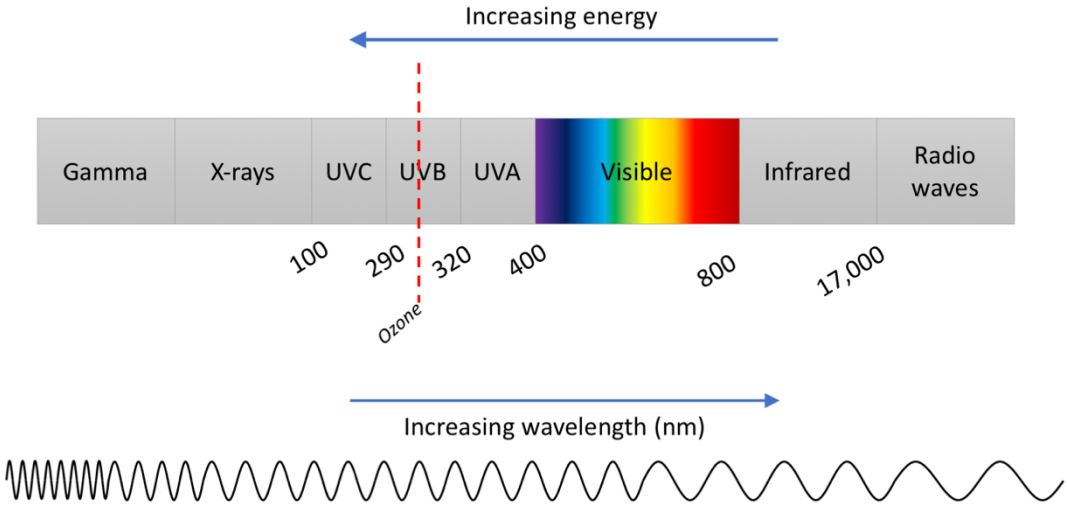


Figure 1.1 The electromagnetic spectrum of radiation. This illustration is adapted from a figure created by Philip Ronan and Gringer released under a CC-BY-SA-3.0 copyright via Wikimedia.

1.1.1 Ambient atmosphere influences the amount of ultraviolet radiation in the immediate environment

The composition of the atmosphere in the immediate environment is heavily influenced by solar altitude and the intensity of sunlight; therefore, variables such as geographic location, season and time of day can all impact on the amount of ambient UVR in the immediate environment (Young, 2009). Moreover, UVR can be reflected, scattered and dampened by atmospheric particles, thus quantities also vary according to the amount of atmosphere UVR must pass through (D'Orazio et al., 2013; Diffey, 2002). As a result, UV concentrations are higher closer to the equator, at higher altitudes and when there is reduced cloud cover (D'Orazio et al. 2013). Also, countries in the southern hemisphere have greater levels of UVR compared to those in the northern hemisphere, as the Earth's orbit brings the southern hemisphere closer to the sun during the summer months (Gies et al. 2004).

An ongoing public health concern is the depletion of the stratospheric ozone layer through excessive production of chlorofluorocarbons (CFCs). Living in an industrialised society has led to increased quantities of CFCs in the atmosphere, contributing to the detrimental chemical breakdown of the Earth's protective shield (Diffey, 2003; Laube et al., 2014; Lucas et al., 2006). Consequently, the amount of UVR reaching the Earth has significantly increased. Despite the fact that cloud cover and low atmospheric pollution make it difficult to assess fluctuations in UVR levels, monitoring of the Swiss Alps where the atmosphere is much clearer, indicates that there are increased levels of UVR in the northern hemisphere (Willemse and Furger, 2016). A similar observation has also been recorded in the southern hemisphere through monitoring UVR levels in Australia (Gies et al., 2004). Increases in ambient UVR pose a serious health threat as it increases the risk of negative health consequences from overexposure.

1.1.2 Electromagnetic energy of solar radiation is biologically active

Although UVR covers a small portion of the electromagnetic spectrum, spanning 100-400nm, the biological effects vary enormously between wavelengths. Thus, UV energy is further divided into UV-A (320-400nm), UV-B (280-320nm) and UV-C (200-280nm) regions (D'Orazio et al., 2013; Diffey, 2002). From solar radiation, only UV-A and UV-B wavelengths are relevant in today's climate as the ozone layer and atmospheric oxygen completely filter out UV-C wavelengths. Much of the UV-B wavelengths are also filtered, resulting in the majority (94-97%) of ambient UVR being comprised of UV-A, while the remaining 6-3% consists of UV-B (Lucas et al., 2006). Both UV-A and UV-B wavelengths penetrate cutaneous tissue of humans and can be absorbed by cellular components; including nucleic acids, urocanic acid, amino acids and melanin precursors. The amount of UVR absorbed through the skin critically influences the biological effects (D'Orazio et al., 2013; Hönigsmann, 2002).

Absorption of UV photons initiates biological reactivity, as photon energy carried in each wavelength is transferred to molecules within cells, causing chemical changes (Diffey and Kochevar, 2007). Each UV absorbing molecule, such as amino acids, nucleotides and porphyrins within skin tissue, are typically referred to as chromophores. These molecules absorb a unique combination of wavelengths, which could include either UV-A/UV-B or both (Young, 1997). Differences in the absorption characteristics of chromophores account for the diverse effects produced at different wavelengths (Diffey and Kochevar, 2007; Lee et al., 2013; Slominski et al., 2018). UV-B wavelengths are the most efficient at exerting biological alterations as the absorption spectra of many cellular structures, including DNA and urocanic acids are within the UV-B range (Budden and Bowden, 2013). This also means that UV-B photons are the most harmful. The efficiency of DNA, RNA and cellular proteins to absorb UV-B photons results in nearly all UV-B

radiation being absorbed within the upper layers of the epidermis (Holick, 2016). These cellular chromophores are less effective at absorbing UV-A radiation, therefore, UV-A penetrates more deeply through the skin, reaching the upper portion of the dermis. UV-A has a tenfold reduced efficiency compared to UV-B, evident by the difference in minimal erythema dose, which is the amount of radiation that will produce minimal erythema (redness) of an individual's skin (Heckman et al., 2013; Slominski et al., 2018). This large difference in magnitude of biological effect has been suggested to arise due to differences in the quantum mechanics of electron excitation within specific chromophores, as the photon energy from UV-A and UV-B wavelengths are similar (UV-A=3.10-3.94nm, UV-B is 3.94-4.43nm) (Slominski et al., 2018). UV-C is profoundly mutagenic and would mostly be absorbed by chromophores within the upper layers of the epidermis, if UV-C was not blocked out by the ozone layer (Lee et al., 2013).

When a biomolecule absorbs UVR it transforms from a "ground state" to an "excited state", which is higher in energy. This is characterised by a change in the electron distribution surrounding the nuclear framework. Each molecule can only absorb photons with certain energies as the laws of quantum mechanics only allow specific energy gaps between the ground state and excited state (Diffey and Kochevar, 2007). As the energy of the photon is inversely related to wavelength, each chromophore has a unique absorption spectrum. The duration of the excited state is very brief and molecules will quickly return to the ground state by giving off light or heat energy or by undergoing a photochemical reaction. A photochemical reaction results in the creation of a photoproduct, as the chromophore is converted to a new molecule that now has a different structure (Holick, 2011). An example of a photochemical reaction is the conversion of 7-dehydrocholesterol (7-DHC) to pre-vitamin D3, in which UV photons alter the structure of the carbon-carbon bonds (Holick, 2011).

1.2 Cutaneous responses to ultraviolet radiation exposure

1.2.1 The structure and function of human skin

The major recipient of UV energy is the skin. hence downstream systemic UV related changes likely arise from skin-messenger systems (Slominski, 2015). Skin tissue is the largest organ of the human body and is anatomically divided into two regions that are separated by a basement membrane (Swann, 2010). The outermost layer, sitting on top of the basement membrane, is the highly cellular epidermis (approximately 100-150μm) forming a protective barrier over the body's surface. Directly beneath lies the dermis (2-4mm), which is predominantly non-cellular but contains structural proteins such as collagen and elastin. The dermis also consists of cutaneous structures including hair follicles, nerves, sebaceous glands and sweat glands, as well as a small number of immune cells and fibroblast cells (D'Orazio et al., 2013; Lugo et al., 2011). Below the dermis lies the hypodermis which is predominately composed of subcutaneous fat and houses the blood and lymphatic vessels destined for the skin (Hayward and Keatinge, 1981).

The main cell type of the epidermis are keratinocytes (95%), which continually proliferate by division at the stratum basale (basal layer) on top of the basement membrane (Pincelli and Marconi, 2010). Non-proliferating keratinocytes transit through a series of well-defined layers, with cells progressing to become anucleated, keratin filled corneocytes that form the outer non-living layer, the stratum corneum (Figure 1.2). The highly stratified cells of the stratum corneum are joined by rigid tight junctions providing the major component of the skin's physical barrier (Proksch et al., 2008). Normal epidermal turnover is under tight homeostatic control where desquamation (cell shedding) is balanced with cell division (Young, 1997). The DNA of keratinocytes is a major chromophore of UV-B, and repeated exposure results in mutations that contribute to the formation of non-melanoma

skin cancers (Nasti and Timares, 2012). Melanocytes and Langerhans cells are the next two prominent cell types of the epidermis, but exist in much smaller quantities. Melanocytes are also found within the basale layer and synthesise melanin pigments through melanogenesis. Melanin pigments are transferred to adjacent keratinocytes as melanosome particles where they contribute to skin colour. Melanin also provides the skin's natural defence against UVR, absorbing UVR photons and dissipating the energy as heat (Lin and Fisher, 2007). Increased sun exposure results in increased melanin production, to protect against sun damage, better known as tanning (Koch et al., 2006; Videira et al., 2013). Langerhans cells are the immunocompetent cells of the skin located throughout the epidermis but most prominently within the stratum spinosum, directly above the stratum basale. Langerhans cells are the antigen presenting cells (APC) of the skin, with long dendritic structures, they provide the first line of immunological defence. Once Langerhans cells become activated, they migrate to skin draining lymph nodes and initiate the proliferation of T cells (Bennett et al., 2007).

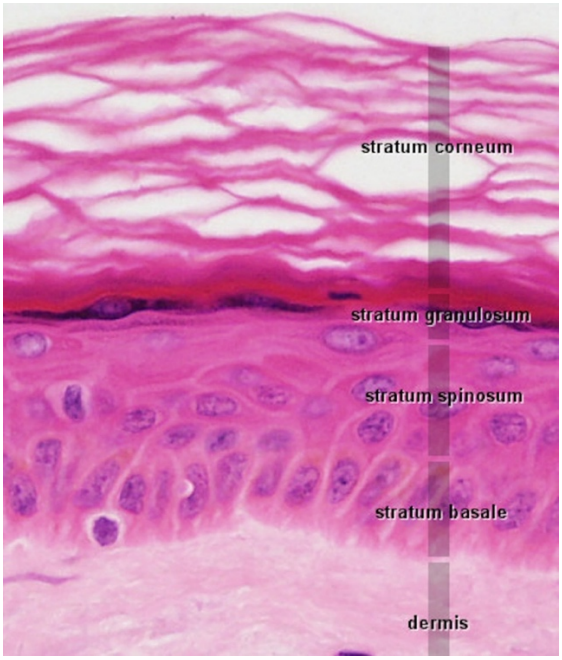


Figure 1.2. Histological section of human skin. The epidermis is divided into the stratum corneum, stratum granulosum, stratum spinosum and stratum basale (Hill, 2018).

1.2.2 Negative consequences of ultraviolet radiation exposure

Sun-related health consequences have been closely examined, as UVR is known to have profound effects on skin physiology. One of the most immediate, acute responses to UVR exposure is erythema, or redness of the skin, which is a radiation type burn, also known as sunburn (Clydesdale et al. 2001). The sunburn response is highly inflammatory, driven by a cascade of cytokines, chemokines, vasoactive and neuroactive mediators that together facilitate vasodilation and cellular repair pathways (D'Orazio et al., 2013; De Gruijl, 1999; Ichihashi et al., 2003; Jans et al., 2006; Matsumura, 2002). If UVR doses exceed the threshold damage response, keratinocytes will activate apoptotic pathways resulting in cell death (Jans et al., 2006; Laethem et al., 2005).

As mentioned previously, the UVR spectrum has distinct pathways of cell damage determined by wavelength. UV-B is responsible for direct damage as it is absorbed by cellular chromophores and therefore has the potential to critically interfere with processes necessary for cell viability (Sklar et al., 2012). DNA is one of the major UV-B chromophores, and damage arises through UV-B-induced cross-linking of pyrimidine bases, thymine and cytosine, resulting in DNA mutations. Double stranded RNA can also be affected and form uracil dimers. The most common DNA photoproducts are cyclobutene pyrimidine dimers and 6,3-pryimidine-pyrimidones. The accumulation of photoproducts alters the structure of DNA, consequently inhibiting DNA polymerases and arresting cell replication (Goodsell, 2001; Sklar et al., 2012; Zhao et al., 2010). Photo-reactivation and nucleotide excision repair can remove dimers from the DNA strand, however, the unrepaired dimers become mutagenic. It is believed that if certain genes, such as the p53 tumour supressing gene, are affected this can lead to unregulated hyper-proliferation of epidermal cells and actinic keratosis. Resulting in the formation of mutated cells that

undergo unregulated cellular differentiation, and can culminate in the formation of a skin cancer (Brash et al., 1991).

UV-A radiation contributes to indirect photodamage through the formation and build-up of reactive oxygen species (ROS) leading to oxidative stress (Salmon et al., 2004). Free radical formation can also lead to DNA damage and increase the risk of skin cancers, as UVR penetrates into the layers of the dermis. Free radicals are responsible for the cross-linking of collagen and elastin fibres, resulting in sun damage and the formation of wrinkles (Schuch et al., 2017). UV-A may also have an effect of the immune system, increasing immune tolerance (Holick, 2016). Continued UVR exposure leads to adaptive skin responses such as increased dermal thickening and increased melanin production, aimed at reducing UV-induced harm (Schuch et al., 2017).

1.2.3 Health benefits of ultraviolet radiation exposure

The health benefits associated with UVR exposure have largely been attributed to photocutaneous production of vitamin D. The same part of the UV spectrum primarily responsible for DNA damage is also required for vitamin D photosynthesis. Thus, the deleterious and beneficial health effects of UV irradiation are inseparable intimately connected (Holick, 2011).

During exposure to sunlight, epidermal 7-DHC absorbs UV-B wavelengths and is converted into the secto-steroid pre-vitamin D3, following a conformational change which breaks the B ring (Bikle, 2014; Zhang and Naughton, 2010). Pre-vitamin D3 then isomerises to D3 or, with continued UV irradiation, to by-products tachysterol and lumisterol (preventing overproduction of vitamin D and toxicity) (Holick et al., 1981). The inactive D3 exists the skin, bound to vitamin D binding protein (VDB), where it must undergo hydroxylation to become metabolically active (Juzeniene et al., 2011). The best

characterised pathway of vitamin D metabolism involves hydroxylation steps in the liver and then the kidney, however, numerous tissues have now been identified to contain the enzymes necessary for conversion of D3 to active vitamin D (calcitriol; 1,25 hydroxycholecalciferol) (Bikle, 2000).

Biologically activity vitamin D exerts it effects through binding to the vitamin D receptor (VDR), primarily located in the nuclei of target cells, which modulates gene transcription (Hossein-nezhad et al., 2013). Therefore, vitamin D influences several metabolic processes, including DNA repair, antioxidant activity and regulating cell proliferation and differentiation (Hossein-nezhad et al., 2013; Montecino et al., 2008). The VDR is found in nearly all organ systems, including the brain, indicating the widespread action of vitamin D on both physiological and pathological processes (Stocklin and Eggersdorfer, 2013).

Sunlight is the major source of vitamin D for most people, contributing approximately 90% of the bioavailable vitamin D within body (Samanek et al., 2006). As such, eliminating sun exposure entirely would be detrimental to human health (Holick, 2011; Wacker and Holick, 2013). Although vitamin D can be acquired through the diet, few foods contain enough vitamin D to maintain adequate levels (Holick, 2009). Deficiency of vitamin D, particularly in early life, has long been associated with poor bone health and development of Rickets or osteomalacia through malabsorption of calcium and phosphorus (Holick, 2011). However, in recent years, studies have demonstrated many non-classical roles for vitamin D in the immune, cardiovascular, muscular, reproductive and integumentary systems, as well as protecting against several cancers (Bikle, 2014; Garland et al., 2006; Norman, 2008; Zhang and Naughton, 2010). Although, randomised controlled trials do not consistently support the findings from observational and pre-clinical studies

that report an association between vitamin D status and reduced risk of disease. This begs the question as to whether vitamin D is a mere proxy for sun exposure. At present, research has been unable to distinguish if UV-related health benefits are solely attributable to vitamin D, or if other UV factors may be in play.

1.2.4 New challenges of ultraviolet radiation related to skin pigmentation in the modern world

The development of different skin pigmentation of humans has likely arisen due to selection pressures associated with exposure to UVR (Jablonski and Chaplin, 2017). This includes, for example, the migration of humans from areas of high ambient UVR to areas of lower ambient UVR, the contrasting requirements of photo protection, and the necessity to receive sufficient sunlight for cutaneous vitamin D production (Jablonski and Chaplin, 2010). Thus, people that inhabit regions closer to the equator, with higher UVR intensity, have darker skin pigmentation for protection from harm caused by UVR; while those at higher latitudes, with much less ambient UVR, have developed fair skin to maximise the production of vitamin D (Brenner and Hearing, 2008; Jablonski and Chaplin, 2010). However, in the modern world, this is not always the case. Rapid human migration in the last few hundred years, out of the areas in which our ancestors evolved, has meant that skin pigmentation is not necessarily suited to the environment in which we live. Dark-skinned populations migrating to areas of higher latitude has led to increased prevalence of conditions attributed to vitamin D deficiency (Kift et al., 2013; Pal et al., 2003). In contrast, fair-skinned populations that have migrated to areas with much higher UVR levels than where their ancestors evolved have experienced rapid increases in the incidence of skin cancers (Armstrong and Kricker, 2001; De Gruijl et al., 2001).

For this reason, people of the modern world are facing new challenges regarding sun avoidance and sun exposure in an environment they have not yet appropriately adapted to. This has resulted populations of people receiving more UVR than ever before, which could be contributing to yet unidentified pathological conditions, such as increased addictive-like behaviours observed in frequent tanners (Chapter 2) and increased incidence of skin cancers. Furthermore, at the opposite end of the spectrum, other people are not receiving sufficient sun exposure, and this appears to be correlated with an increased risk of chronic illness, infectious disease and increased mortality, assumedly due to insufficient vitamin D (Hoel et al., 2016). As such, the environmental burden of disease attributed to UVR is relevant across the globe, and UVR is concomitant with life on Earth. Thus, highlighting the need for ongoing research on the physiological responses to UVR.

1.3 Skin-brain axis: How ultraviolet radiation "touches" the brain

UVR exposure has been shown to affect mood, addictive behaviour, cognition and memory, indicating that external UVR can access neuronal circuitry and influence behavioural outcomes (Beecher et al., 2016; Dominiak et al., 2015; Fell et al., 2014; Keller et al., 2005). The principle pathway by which UV light applied to the skin has been thought to contribute to modulation of neuronal circuitry is via the actions of vitamin D (Holick, 2011). However, recent developments suggest other systems, such as the neuroendocrine system, could be contributing to UV-induced modulation of behaviour (Skobowiat et al., 2017; Slominski, 2015). This section will therefore explore the emerging evidence of potential pathways by which UVR exposure to the skin contributes to downstream modulation of neuronal circuitry.

A growing body of literature suggests that UVR, through skin neuroendocrine systems (i.e. via the circulation and/or activating ascending neural pathways), can signal to

the brain; facilitated by the skin analogue of the hypothalamic-pituitary-adrenal (HPA) axis (Slominski et al., 2018; Zmijewski and Slominski, 2011). Organisation of this local "equivalent" HPA axis follows the same activation pattern as the central HPA axis, culminating in the production of glucocorticoids by the adrenal glands (Slominski et al., 2013). In brief, noxious stimuli or stressors (such as UVR) are detected by skin cells, which enhance the cutaneous production or release of corticotrophin releasing hormone (CRH) from nerve endings (Slominski et al., 2001; Slominski et al., 2013). In turn, CRH triggers the subsequent secretion of adrenocorticotrophic hormone (ACTH) from the pituitary gland (Grammatopoulos and Chrousos, 2002; Slominski et al., 2013). ACTH, then circulates to the adrenal cortex contributing to the release of glucocorticoids, through the activation of glucosteriodogenesis from cholesterol (Miller and Auchus, 2011). Glucocorticoids can easily access the brain where they bind to receptors to influence neuronal signalling and behaviour, especially through altered glutamate neurotransmission in the prefrontal cortex and hippocampus, thereby influencing cognitive and emotional processing (Lupien et al., 2009; Popoli et al., 2011). Experimental research in mice demonstrates that when UV-B is applied directly to the skin it triggers activation of both the local and central HPA axis, resulting in elevated levels of CRH, urocortin, ACTH, β-endorphin and corticosterone in plasma and brain tissue (Skobowiat et al., 2011; Skobowiat and Slominski, 2015). This indicates that UVR exposure is capable of activating a systemic stress response, centred in the HPA axis, which is initiated by skin cells (Skobowiat and Slominski, 2015). More recently, UV-B exposure has also been shown to activate the proopiomelanocortin (POMC) signalling pathway in the arcuate nucleus of the hypothalamus (Skobowiat and Slominski, 2016). POMC is a precursor protein of numerous neuropeptides, including ACTH, α-melanocyte stimulating hormone (α-MSH) and β-endorphin. Within the arcuate nucleus, these neuropeptides play a role in the regulation of feeding behaviour (Dutia et al., 2012).

Following UV-B application, dose-dependent increases in POMC gene expression, accompanied by increased plasma levels of α-MSH and β-endorphin, were observed in two genetically different strains of mice (Skobowiat and Slominski, 2016). Together, these findings emphasise that the brain is involved in translating peripheral UVR signals into systemic responses.

Cutaneous exposure to UVR also contributes to alterations in the levels of peripheral proteins, peptides and small molecules, including vitamin D, melanin, β-endorphin and nitric oxide (NO), both locally and within the circulation (Brenner and Hearing, 2008; Fell et al., 2014; Hart et al., 2011; Holliman et al., 2017). These small molecules, through similar mechanisms involving the circulation and/or ascending neuronal pathways, may likewise signal to the brain and influence neuronal signalling to alter behaviour. Preclinical studies have demonstrated that cutaneous UVR exposure contributes to elevated levels of β-endorphin (generated as a by-product in the cleavage of POMC), resulting in opioid-related increases in nociceptive thresholds. This effect could be reversed with the opioid antagonist naloxone, and was abated in β-endorphin null mice (Fell et al., 2014). Recently, another study demonstrated that UVR triggered elevation of uranic acid in the blood, which in turn crossed the blood brain barrier (BBB), promoting the biosynthesis and release of glutamate within several areas on the brain (Zhu et al., 2018). UVR exposure was subsequently correlated with enhanced performance in motor learning and recognition memory tasks in mice; an effect that could be blocked with the application of dipeptide glycyl-glycyl inhibiting urocanase within the neuron or with short hairpin RNA, preventing the enzymatic actions of urocanase (Zhu et al., 2018). This evidence further suggests a connection between peripheral and central systems invoked by UVR exposure on the skin.

1.4 The innate immune system facilitates peripheral-to-central communication

The idea that events in the periphery, particularly those of an inflammatory nature, have profound influence on brain function has been widely accepted (for reviews see (Capuron and Miller, 2011; Dantzer et al., 2008; Quan and Banks, 2007; Watkins and Maier, 2000). Critically involved in host defence against invading pathogens, the innate immune system also has the ability to respond to endogenous factors initiated from cell stress/damage (Maier, 2003). Thus, the innate immune system is a likely candidate to facilitate cross-talk between the skin and the brain following UVR exposure.

Virtually all organs of the human body contain immunocompetent cells that express pattern recognition receptors (PRRs) for the detection of damage associated molecular patterns (DAMPs) released during tissue injury or infection (Bilbo and Schwarz, 2012). DAMPs are biomolecules that initiate and perpetuate inflammatory signalling cascades necessary for host defence. In response to inflammatory stimuli, activation of these signalling cascades result in bidirectional communication between the periphery and the brain (immune-to-brain communication), alerting higher centres to danger to implement an appropriate host response (Maier, 2003). Within the CNS, this process is mediated by glia cells which are a population of non-neuronal support cells (previously regarded as inert) that are now recognised to have an integral role in maintaining homeostasis and can influence neurotransmission (Ousman and Kubes, 2012). The major resident immune-like cells of the CNS are microglia, which together with astrocytes and the pre- and post-synaptic neuron, form the tetrapartite synapse (De Leo et al., 2006). This structure is a key site of interaction in which each cell reciprocally signals to one another, enabling glial cells to mediate neuronal signalling and contribute to "neuroimmune communication". The close proximity of the tetrapartite synapse means glial cells rapidly respond to disruptions in homeostasis, having a marked effect on neuronal signalling and behavioural adaptations;

for example, lethargy, anhedonia and fever experienced during times of sickness (Dantzer, 2004; Kelley et al., 2003). However, as the periphery is virtually segregated from central tissue by the BBB, a physical impediment that restricts free access into the brain, immune-to-brain communication relies on a network of highly sophisticated cell interactions involving hormones, neurotransmitters, and cytokines (Quan and Banks, 2007).

So far, several mechanisms, acting in parallel, have been identified by which peripheral signals can access the brain (Figure 1.3). First, evidence suggests that blood-borne cytokines can passively diffuse into the brain via regions lacking a contiguous BBB, including the circumventricular organs and the choroid plexus (Breder et al., 1988; Komaki et al., 1992; Saper and Breder, 1994). The close proximity and bidirectional neuronal projections from the circumventricular organs to the hypothalamus (McKinley et al., 1994; Silverman et al., 1981), hippocampus (Ciriello and Gutman, 1991) and amygdala (Silverman et al., 1981) suggest that circulating immune signals may be further projected into the brain via these connections (Quan, 2008). Secondly, multiple endothelial-related mechanisms by which peripheral signals may enter the brain through an intact BBB have been identified. These include energy-dependent transport systems in which cytokines such as interleukin (IL)-1, IL-6 and tumour necrosis factor (TNF) access the brain via saturable transport systems. Yet, it remains unclear whether the quantity of cytokines transported is sufficient to mediate behavioural adaptions (Banks et al., 1991). Peripheral leukocytes may cause increased immune signalling within the CNS, without physically crossing the BBB, through interactions with endothelial cells of the cerebral vasculature (D'Mello et al., 2013). Alternatively, adhesion molecules, such as ICAM (intracellular adhesion molecules) and VCAM (vascular adhesion molecules) allow passage of signals through the tissue (Wong et al., 2007). Endothelial cells also have the capacity to release immune-related molecules such as NO, prostaglandins, IL-1, and IL-6 (Fabry et al., 1993). Thus, blood-derived

cytokines may initiate release of cytokines into the CNS through peripheral binding, without physically entering. The aforementioned humoral and cellular pathways of immune-to-brain communication are dependent on blood-derived stimuli, circulating to the proximity of the CNS to initiate central neuroinflammatory processes. The final identified pathway provides a much faster mode of transmission, relaying information via the autonomic nervous system (Bluthe et al., 1994; Dantzer et al., 1998; McCusker and Kelley, 2013). Locally produced cytokines are thought to directly stimulate primary afferent nerve fibres, such as the vagal nerve during abdominal or visceral infections, or the trigeminal nerve during orolingual infections. Signalling from these nerves in turn rapidly activates central pathways involved in sickness behaviours (Bluthe et al., 1994; Dantzer et al., 1998; McCusker and Kelley, 2013). Overall, these convergent pathways culminate in the production of inflammatory cytokines and the activation of glial cells within the brain, generating neuroinflammation, which is distinct from its peripheral source. This is a critical point to highlight as it is the presence of central cytokines and their direct contact with neuronal targets, facilitated by the tetrapartite synapse between glia and neurons, that elicits behavioural responses; regardless of the originating peripheral cytokine signals (Eyo and Wu, 2013; De Leo et al., 2006).

The existence of these bidirectional communication pathways between peripheral and central tissues creates the opportunity for immune signalling to have broad implications on processes not normally associated with immune function. Furthermore, the potential for the immune system to manipulate neuronal circuits paves a pathway by which peripheral cutaneous UVR, which is inflammatory at both UV-A and UV-B wavelengths, could result in behavioural adaptions by affecting central circuitry. The relationship between cutaneous UVR and its effect on behaviour, potentially mediated by the immune system, has been

comprehensively reviewed as part of this research project. The following chapter presents this discussion in its peer-reviewed manuscript format.

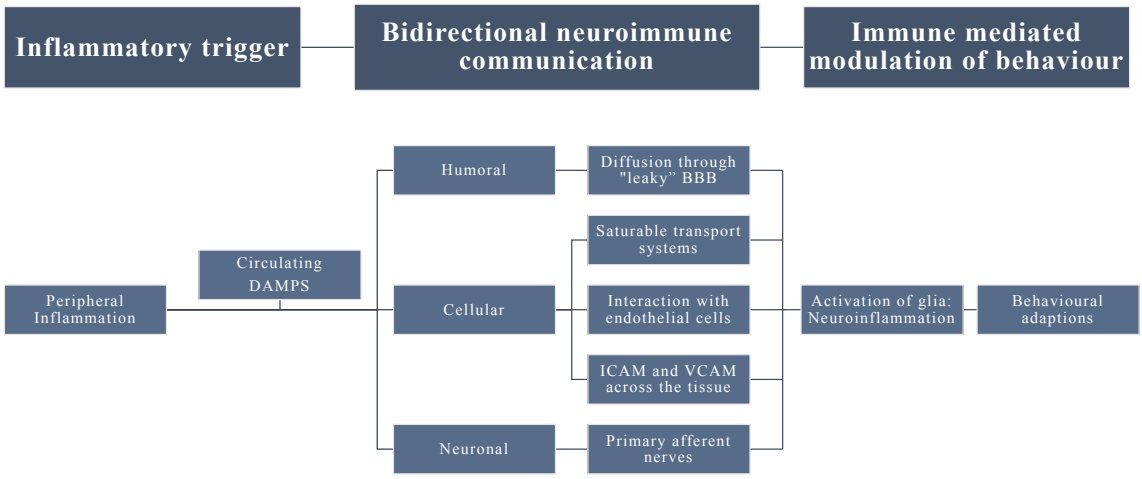


Figure 1.3 Schematic representation of neuroimmune communication. Inflammatory stimuli contribute to the release of circulating DAMPs that facilitate bidirectional communication between the peripheral tissues and the brain. Several pathways have been identified by which peripheral signals may access central tissue to initiate neuroinflammation. A neuroinflammatory state can directly modulate neuronal transmission, via the tetrapartite synapse, resulting in immune-mediated behavioural adaptions.

Statement of Authorship

Title of Paper	Can neuroimmune mechanisms explain the link between ultraviolet light (UV) exposure and addictive behaviour?
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Iacopetta, K., et al. (2018). "Can neuroimmune mechanisms explain the link between ultraviolet light (UV) exposure and addictive behavior?" Brain Behav Immun(73): 125-132.

Principal Author

Name of Principal Author (Candidate)	Krystal Iacopetta		
Contribution to the Paper	Manuscript conception, reviewed all papers cited in the manuscript, wrote manuscript, designed figures and acted as corresponding author.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	18/12/2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Lyndsey E. Collins-Praino		
Contribution to the Paper	Supervisor, assisted with preparation and editing of manuscript.		
Signature		Date	18/12/2018

Name of Co-Author	Femke T.A Buisman-Pijlman		
Contribution to the Paper	Supervisor, assisted with preparation and editing of manuscript.		
Signature		Date	18/12/2018

Name of Co-Author	Mark R. Hutchinson			
Contribution to the Paper	Supervisor, assisted with preparation and editing of manuscript.			
Signature			Date	18/12/2018

<u>Chapter 2.</u> Can neuroimmune mechanisms explain the link between ultraviolet light exposure and addictive behaviour?

This chapter has been previously published in a peer-reviewed journal [Iacopetta K et al. (2018) *Brain, Behaviour and Immunity*].

This thesis explores the relationship between UVR-induced skin signalling and its influence on brain function, asking the question of how UVR influences brain neuro circuitry in health and disease. In the following review paper, we have endeavoured to highlight that UVR seeking behaviour is comparable to other behavioural addictions, and that UVR exposure results in engagement of systems, such as the opioid and dopamine system, that are critical in the development of addiction. The concept of neuroimmune communication and its role in the development of addiction is introduced, and a novel hypothesis of alarmin-derived skin-brain signalling is presented.

ARTICLE IN PRESS

Brain, Behavior, and Immunity xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Review Article

Can neuroimmune mechanisms explain the link between ultraviolet light (UV) exposure and addictive behavior?

Krystal Iacopetta[a], Lyndsey E. Collins-Praino[a], Femke T.A. Buisman-Pijlman[a], Mark R. Hutchinson[a,b,*]

[a] Adelaide Centre for Neuroscience Research, Adelaide Medical School, University of Adelaide, SA, Australia
[b] Australian Research Council Centre of Excellence for Nanoscale BioPhotonics, University of Adelaide, Adelaide, SA, Australia

ARTICLE INFO

Keywords:
Alarmins
Tanning addiction
Neuroimmunology
Dopamine
Inflammation

ABSTRACT

High ultraviolet (UV) light exposure on the skin acts as a reinforcing stimulus, increasing sun-seeking behavior and even addiction-like sun seeking behavior. However, the physiological mechanisms that underlie this process remain to be defined. Here, we propose a novel hypothesis that neuroimmune signaling, arising from inflammatory responses in UV-damaged skin cells, causes potentiated signaling within the cortico-mesolimbic pathway, leading to increased sun-seeking behaviors. This hypothesized UV-induced, skin-to-brain signaling depends upon cell stress signals, termed alarmins, reaching the circulation, thereby triggering the activation of innate immune receptors, such as toll-like receptors (TLRs). This innate immune response is hypothesized to occur both peripherally and centrally, with the downstream signaling from TLR activation affecting both the endogenous opioid system and the mesolimbic dopamine pathway. As both neurotransmitter systems play a key role in the development of addiction behaviors through their actions at key brain regions, such as the nucleus accumbens (NAc), we hypothesize a novel connection between UV-induced inflammation and the activation of pathways that contribute to the development of addiction. This paper is a review of the existing literature to examine the evidence which suggests that chronic sun tanning resembles a behavioral addiction and proposes a novel pathway by which persistent sun-seeking behavior could affect brain neurochemistry in a manner similar to that of repeated drug use.

2.1 Abstract

High ultraviolet (UV) light exposure on the skin acts as a reinforcing stimulus, increasing sun-seeking behaviour and even addiction-like sun-seeking behaviour. However, the physiological mechanisms that underlie this process remain to be defined. Here, we propose a novel hypothesis that neuroimmune signalling, arising from inflammatory responses in UV-damaged skin cells, causes potentiated signalling within the cortico-mesolimbic pathway, leading to increased sun-seeking behaviours. This hypothesised UV-induced, skin-to-brain signalling depends upon cell stress signals, termed alarmins, reaching the circulation, thereby triggering the activation of innate immune receptors, such as toll-like receptors (TLRs). This innate immune response is hypothesised to occur both peripherally and centrally, with the downstream signalling from TLR activation affecting both the endogenous opioid system and the mesolimbic dopamine pathway. As both neurotransmitter systems play a key role in the development of addiction behaviours through their actions at key brain regions, such as the nucleus accumbens (NAc), we hypothesise a novel connection between UV-induced inflammation and the activation of pathways that contribute to the development of addiction. This paper is a review of the existing literature to examine the evidence which suggests that chronic sun tanning resembles a behavioural addiction and proposes a novel pathway by which persistent sun-seeking behaviour could affect brain neurochemistry in a manner similar to that of repeated drug use.

2.2 Introduction

Since the mid 20th century, it has become socially desirable and acceptable in many cultures to have the "healthy tan" that comes with sun exposure (Hunt et al., 2012). A tan develops as ultraviolet radiation (UVR) triggers keratinocyte release of α-melanocyte

stimulating hormone (α-MHS) through p53-mediated transcriptional induction of the proopiomelanocortin (POMC) gene, which results in increased synthesis of the pigment melanin (Cui et al., 2007; Videira et al., 2013). Melanin forms a protective barrier to absorb UVR, preventing DNA damage and consequently causing the skin to darken (Bergenmar and Brandberg, 2001). While this may lead to the desired "healthy tan" effect, prolonged and repeated exposure to UV is a public health concern due to its causal relationship to the development of skin cancer (Young, 2009).

The incidence of skin cancer continues to rise in many Westernised nations including the United States, Canada, Australia, New Zealand and numerous countries in Europe (Lucas et al., 2006). The increase of skin cancer has been attributed to a multitude of factors, some of which fall outside the locus of control of the individual, such as effects of pollution damaging the ozone layer, increasing the amount of ambient UVR (Diffey, 2003; Leary and Jones, 1993). However, the single biggest risk factor continues to be behavioural: the willingness to be exposed to UVR for the aesthetic purposes of achieving a tanned appearance (Diffey, 2003; Leary and Jones, 1993). These behavioural choices can include increased voluntary UVR exposure through sunbathing, an increased amount of time spent outdoors participating in leisure activities, and changes in clothing styles exposing more skin (Diffey, 2003; Leary and Jones, 1993).

The desire to tan has largely been attributed to appearance factors acting as the primary reinforcement; that is, people believe they look and feel better with a tan (Dennis et al., 2009). However, an increasing body of literature is emerging indicating that UVR itself is a reinforcing stimulus, acting to increase UV-seeking behaviours (Feldman et al., 2004; Fell et al., 2014; Harrington et al., 2011a). Despite increasing efforts to alter perceptions of tanning and increase awareness of the damaging effects of UVR, frequent tanners are not deterred from engaging in tanning behaviour (Montague et al., 2001),

particularly among adolescents and young adults (Cokkinides et al., 2006; Guy et al., 2015; Livingston et al., 2003). In fact, although public knowledge regarding the dangers of sun exposure greatly increased between 1986 and 1996, sun burning and regular use of tanning beds also increased (Robinson et al., 1997).

Remarkably, survey studies have revealed that frequent tanners are well informed, often displaying higher levels of knowledge regarding the risk of UVR than their non-tanning peers (Poorsattar and Hornung, 2007). An overwhelming majority, more than 90% of past and current tanners surveyed, revealed that they believed that skin cancer was a possible consequence of tanning. The same study also identified that 81% of past tanners and 53% of current tanners did not believe that tanning beds were safe (Knight et al., 2002), indicating that tanners know the dangers of tanning. Frequent tanners are more likely to be informed about UV-induced skin damage but are also more likely to believe that tanned skin improves appearance and protects against subsequent UV damage (Grange et al., 2015). Even having a hereditary risk of cancer was not enough to prevent the use of tanning beds in a sub-population of young adults (Bergenmar and Brandberg, 2001).

Therefore, despite a growing awareness of the danger associated with overexposure to UVR and the increased risk of skin cancer or having watched a family member face a deadly cancer, frequent tanners are not deterred from engaging in tanning behaviour. In this way, continuing to tan resembles behaviours associated with substance use or gambling disorder when there is a loss of control of a behaviour despite being aware of the potential dangers. The person is not able to change the behaviour even when adverse consequences arise that are clearly aggravated by the use (American Psychiatric Association, 2013; Kourosh et al., 2010; Nolan and Feldman, 2009).

While the exact mechanisms that may lead to tanning dependence are still unknown, this paper reviews the evidence that chronic UV exposure has physiologically reinforcing

effects comparable to addiction and proposes a novel hypothesis by which UV light could affect brain neurochemistry via cutaneous-brain inflammatory signalling.

2.3 Evidence to suggest that tanning is comparable to behavioural addictions

People who tan frequently can exhibit signs of psychological and physical dependence that parallel those seen in other addictive disorders suggesting a form of behaviour addiction (Poorsattar and Hornung, 2007; Stapleton et al., 2017). For example, using diagnostic screening tests, such as the CAGE (cut down, annoyed, guilty, eye-opener) questionnaire (Ewing, 1984), originally developed for alcohol dependence, as well as criteria from the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR) adapted for tanning behaviour, it was discovered that approximately 70% of both frequent outdoor (Warthan et al., 2005) and indoor (Harrington et al., 2011b) tanners met the modified criteria for addictive disorders or dependence. Specifically, frequent tanners continued to tan despite attempts to stop, showed persistent tanning in the presence of adverse consequences and neglected other responsibilities to maintain a tan (Harrington et al., 2011b; Warthan et al., 2005). Collectively, these behaviours show significant resemblance to the characteristics that define addictions such as gambling disorder (American Psychiatric Association, 2013). In support of this, a survey of 325 American college students, using the Structured Interview for Tanning Abuse and Dependence (SITAD), discovered that 10.8% and 5.4% of this population met criteria for tanning abuse and dependence, respectively. These rates are comparable to those reported for alcohol (5.8%) and alcohol or any illicit drug dependence (7.7%) in this age group (Hillhouse et al., 2012).

Moreover, when we consider individual difference in behaviour it appears that factors such as age of initiation and frequency of the tanning behaviour correlate with

difficulty to cease tanning among adolescents (Zeller et al., 2006), similar to what we see in drug dependence. Those who were 14 to 15 years old at the time of the initiation were more likely to report difficulty in stopping the behaviour compared to 16 to 17 year olds (Zeller et al., 2006). This parallels other high-risk activities, such as gambling, smoking and alcohol abuse, in which age of initiation is inversely correlated with success of quitting (Abdolahinia et al., 2012; Guttmannova et al., 2011; Khuder et al., 1999; Rahman et al., 2012).

Mechanistically, it is important to consider whether there are similarities in the biological basis of the effects of UVR exposure and the development of compulsive tanning with that of behavioural addictions. Frequent tanners indicated that besides aesthetic appeal (90%), tanners wanted to feel good (69%) and relax (56%) (Harrington et al., 2011b). Although the perception of a good appearance may be reinforcing, the latter two responses are comparable to what is reported by those with substance use disorders as reasons for drug or alcohol use (Titus et al., 2007). But are the tanners addicted to UVR exposure, or mainly the relaxing nature of the activity? A study by Feldman et al. (2004) highlighted that frequent tanners show a preference for UV tanning beds compared to identical non-UV beds, based solely on subjective feeling (Feldman et al., 2004). This suggests that the UV light may have physiologically reinforcing effects separate from aesthetic motivation alone.

However, is there any evidence that there is similar engagement of the mesolimbic dopamine or opioid system in frequent tanning, as is seen with other forms of addiction? Dopamine efflux follows administration of amphetamines (Di Chiara and Imperato, 1988), alcohol (Melendez and Rodd-Henricks, 2002) and cannabinoids (Jianping et al., 1990). Increased synaptic dopamine is also implicated in multiple behaviours that can become addictions, such as overeating (Avena et al., 2009), gambling (Bergh et al., 1997) and

sexual behaviour (Balfour et al., 2004). Aubert et al. (2016) showed an increase in dopamine efflux in addicted tanners in response to UVR compared to sham UVR, while such changes were not seen in infrequent tanners (Aubert et al., 2016). Interestingly, a pilot study using SPECT (single photon emission computed tomography) imaging to measure regional cerebral blood flow (rCBF) during a (blinded) session with either UVR or sham UVR in frequent tanners demonstrated that during the UVR session, tanners had a significant increase in rCBF in the left striatum, indicating activation of this dopamine rich region (Harrington et al., 2011a). These changes were accompanied by a decrease in the subjective desire to tan. Similarly, emerging evidence suggests that the reinforcing effects of UVR may be mediated by the opioid system, specifically through β-endorphin (Fell et al., 2014; Kaur et al., 2005; Kaur et al., 2006). β-endorphin is an endogenous opioid peptide with high affinity for the μ-opioid receptor (Volkow and McLellan, 2016), the same receptor responsible for producing the analgesic and euphoric properties from exogenous opioid derivatives, including drugs of abuse (Akil et al., 1998). UVR exposure in rodents, as well as cultured human cells, triggers release of β-endorphin as a by-product of the physiological processes that contribute to tanning (Fell et al., 2014). Daily exposure to UVR for a period of 6 weeks was associated with elevated plasma levels of β-endorphin in rodents. This was accompanied by increases in pain related thresholds, an effect that could be reversed with systemic administration of opioid antagonist, naloxone, and was abated in β-endorphin null mice. Administration of naloxone following chronic daily UV exposure resulted in numerous signs of opioid withdrawal in mice under experimental conditions. This included increased wet dog shake, paw tremor, teeth chatter and rearing. Furthermore, chronically UV-irradiated mice conditioned with naloxone in a black box developed conditioned place aversion for the black box during the postconditioning preference testing. Conversely, naloxone conditioning in the black box had no effect on mock UV-irradiated

mice, demonstrating that chronic UV exposure imparts an opioid-like physical dependence sufficient enough to guide behaviour. Finally, cross tolerance between UV-exposure and morphine developed, as evidenced by the fact that higher doses of morphine were required to produce comparable analgesia between mice that had received chronic UV-irradiation compared to mock UV (Fell et al., 2014).

Taken together, evidence supports that tanning is a form of behavioural addiction, with similar impacts on brain neurotransmission as other types of addiction. However, the physiological mechanisms that link UVR with an increase in addictive-like behaviour are still unclear. In order to understand this, it is necessary to look at other aspects of addiction pathophysiology, including the role of the immune system in this process.

2.4 Why the immune system is relevant to addiction

A major development in addiction research in recent years has been the discovery that immune signalling within the central nervous system significantly influences signalling pathways responsible for reward processing (Hutchinson and Watkins, 2014). Research by Hutchinson et al. (2012) has demonstrated that a pro-inflammatory state increases the vulnerability to addiction. Inflammatory mediators (e.g. cytokines and chemokines) are able to change the pharmacodynamic actions of drugs (Hutchinson et al., 2008; Narita et al., 2006). For example, morphine reward behaviours are modulated through central immune signalling. Direct injection of an astrocyte-conditioned medium into the nucleus accumbens (NAc) leads to heightened morphine conditioned place preference, indicative of drug preference in rodents (Narita et al., 2006). Conversely, blocking the proinflammatory response from glia prevents morphine and oxycodone conditioned place preference (Hutchinson et al., 2008; Hutchinson et al., 2012).

It has been demonstrated that these effects occur through Toll-like receptors (TLRs), particularly TLR4 (Jacobsen et al., 2014), which are located on the cell membrane of glial cells. Opioid activation of TLR4 contributed to drug reinforcement, as pharmacological blocking of TLR signalling, along with the use of genetic knock out animals, suppressed opioid-induced conditioned place preference and decreased levels of extracellular dopamine (Hutchinson et al., 2012). Similarly, another study demonstrated cocaine interacts with TLR4 and induces pro-inflammatory signalling heightening the rewarding properties of cocaine (Northcutt et al., 2015). Systemic administration of cocaine led to upregulation of pro-inflammatory cytokine interleukin-1β (IL-1β) within the ventral tegmental area (VTA), yet blockade of either TLR4 or IL-1β signalling suppressed cocaine induced increases of dopamine concentrations within the NAc highlighting the importance of TLR signalling in cocaine reinforcement (Northcutt et al., 2015). When a drug of abuse binds at a TLR4 receptor, it facilitates glial activation and increases the production and release of pro-inflammatory cytokines. Pro-inflammatory cytokines are capable of altering neuroexcitability impacting on neuronal output as they upregulate the surface expression of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and NMDA (*N*-methyl-D-aspartate) receptors, increasing spontaneous neurotransmitter release and glutamate transmission (Stellwagen et al., 2005; Viviani et al., 2014). Furthermore, tumour necrosis factor (TNF)-α contributes to endocytosis of $GABA_A$ receptor, resulting in fewer surface receptors and decreased inhibitory strength (Stellwagen et al., 2005). Therefore, TLR4 activity may amplify changes in neuronal activity induced by drugs of abuse, such as increased opioid and dopamine signalling, and contribute to their rewarding properties (Hutchinson et al., 2012; Grace et al., 2014). Taken together, this suggests that the rewarding, and possibly addictive, effects of some drugs require stimulation not only of

neuronal but also of immune-like cell functioning (Coller and Hutchinson, 2012; Hutchinson and Watkins, 2014).

Inflammation also plays a role in the onset and maintenance of binge eating behaviour, which is now listed in the diagnostics for addiction disorders (Corcos et al., 2003; Holden and Pakula, 1996). Binge eating and excessive consumption of certain foods can produce behaviours and changes in the brain that resemble an addiction-like state, involving adaptations to both the dopamine and opioid systems similar to that seen with drugs of abuse (Bello and Hajnal, 2010; Corwin et al., 2011). Abdominal obesity-associated inflammation is suggested to affect neural circuits that may promote addictive behaviours leading to self-perpetuating cycles associated with food intake (Heber and Carpenter, 2011). Hypothalamic levels of IL-18, as well as the IL-18 receptor systems, were down regulated in an animal model of binge eating (Alboni et al., 2017), providing supporting evidence that inflammatory mediators in specific brain regions can contribute to the onset of maladaptive behaviour.

Given the role of the immune system not only in the vulnerability to drugs of abuse, but also in behavioural addictions, such as binge eating, it is reasonable to hypothesise that immune system activation may also be a key factor in the addictive-like behaviours shown by frequent tanners.

2.5 Ultraviolet radiation exposure damages skin cells contributing to release of endogenous danger signals ("alarmins") that activate the immune system

A growing body of evidence demonstrates that UVR damages skin cells, leading to apoptosis, necrosis and pyroptosis (unexpected inflammatory cell death) of epidermal cells following exposure (D'Orazio et al., 2013; De Gruijl, 1999; Ichihashi et al., 2003; Jans et al., 2006; Matsumura, 2002). UV-B wavelengths (290-320nm) are the most cytotoxic to

cells, as they are directly absorbed by DNA (Schuch et al., 2017). The energy from UV-B causes bond opening of DNA nitrogenous bases, allowing adjacent bases to join together via covalent bonding to form a cyclobutane ring of pyrimidine dimers (Jans et al., 2006; Zhao et al., 2010). Single bonds between two carbon atoms and the cyclobutane ring may also form, which results in the "6-4 (T-C) photoproduct" (Zhao et al., 2010). UV-induced single and double strand breaks occur rapidly, and it has been estimated that each skin cell might experience 50-100 reactions per second of exposure (Goodsell, 2001). The disruption of the DNA structure ultimately effects gene sequencing, impacting on cell viability (e.g., transcription and replication) (Sklar et al., 2012). UV-A wavelengths (320-400nm) are less damaging, but can penetrate more deeply, causing indirect photodamage through the creation of reactive oxygen species (ROS) and oxidative stress (Salmon et al., 2004). With increasing levels of UVR and the accumulation of photoproducts cell DNA is compromised, forcing cells to either initiate cellular repair pathways or undergo apoptosis or pyroptosis (inflammatory cell death) (Jans et al., 2006).

Damaged skin cells immediately begin secreting danger signals that "sound the alarm," recruiting immune cells through the release of chemokines and cytokines. Unexpected cell death results in the spillage of intracellular contents into the extracellular space, further exacerbating distress signals (Rock and Kono, 2008). These endogenous mediators are collectively known as "alarmins". Examples of alarmins include adenosine triphosphate (ATP), uric acid, ROS, DNA, and DNA-binding proteins, such as high mobility group box 1 (HMGB1), heat shock proteins (HSPs), inflammatory cytokines and S100 proteins, all of which can be rapidly released from cells during infection or tissue injury, including UVR (Cayrol and Girard, 2014; Chan et al., 2012; Roh et al., 2008).

Numerous studies have shown that alarmins are released from skin cells following stimulation with UVR (Byrne et al., 2011; Johnson et al., 2013; Takai et al., 2011;

Yoshizumi et al., 2008). For example, UV-B radiation induced the release of ATP from HaCat (human keratinocytes) cells (Takai et al., 2011). ATP levels were significantly elevated within the culture medium after 1-minute following 100mkJ/cm^2 of UV light exposure. Release of ATP was determined to occur via maxi-anion channels and anion transporters (Takai et al., 2011). A separate study demonstrated that UV-induced ATP release likely contributes to synthesis of IL-6, as administration of a purinergic (P2) receptor antagonist blocking ATP release inhibited the expression of IL-6 in UV-irradiated normal human epidermal keratinocytes (NHEK) cells (Inoue et al., 2007).

Moreover, acute exposure to UVR (300J/m^2 UV-B) stimulated the release of HMGB1 and pro-inflammatory cytokines, including IL-1β, TNF-α, and IL-6, from human keratinocytes (Johnson et al., 2013). While levels were low in the non-irradiated cells, they began to increase 12 hours post-UV and were highest 24 hours following UV exposure. Similarly, Yoshizumi and colleagues (2008) demonstrated that a low dose of UVR (100mkJ/cm^2) significantly increased concentrations of IL-6, interferon (IFN)-γ and TNF-α, while a higher dose of UVR (400mkJ/cm^2) further compounded the inflammatory response, also increasing the release of IL-1β, granulocyte-colony stimulating factor (G-CSF) and chemokines IL-8 and macrophage inflammatory protein (MIP)-1 from cultured HaCaT cells (Yoshizumi et al., 2008). HMGB1 release from epidermal keratinocytes and corresponding inflammation has also been shown to be elevated in a murine model (Johnson et al., 2013; Yoshizumi et al., 2008).

IL-33 has also been identified as an endogenous alarmin released from murine keratinocytes and dermal fibroblasts (*in vivo)* as well as human skin explants and cells derived from normal human skin (*ex vivo*) (Byrne et al., 2011). Both protein and mRNA expression of IL-33 were increased at 24 hours following exposure to solar simulated UV

light; conversely no expression of IL-33 was observed in non-irradiated skin samples (Byrne et al., 2011).

HSPs are another group of alarmins which are constitutively expressed in human skin under normal conditions, including HSP27, 60, 70, 90, 110 and therefore are assumed to have a critically important role in maintaining fundamental cutaneous processes (Jonak et al., 2009). There is some debate as to whether UV exposure itself, rather than increases in temperature, is responsible for increased expression of HSPs from epidermal keratinocytes and melanocytes (Muramatsu et al., 1992; Muramatsu et al., 1993; Trautinger et al., 1993). A more recent study reported that UV exposure increased expression of HSP70 in cultured human dermal fibroblast, yet the same increase was not observed in the epidermal cell types. Both keratinocytes and melanocytes displayed high baseline levels of HSP70 protein expression but this was not upregulated following UV exposure (Roh et al., 2008). UV-A has been shown to induce HSP72 mRNA and protein expression through what appears to be caused by oxidative damage (Keyse and Tyrrell, 1989; Tyrrell, 2000).

Inflammatory doses of UV trigger cutaneous production of uric acid, as demonstrated in both in vitro and in vivo models (Leighton et al., 2013). Using a murine keratinocyte cell line, doses of 60mkJ/m^2 UV lead to substantial increases in the level of uric acid measured in the supernatant between 1 and 2 hours following irradiation (Leighton et al., 2013). Similarly, in an in vivo model, C57BL/6 mice irradiated with 80mkJ/m^2 of UV had significantly elevated skin levels of uric acid when compared to un-irradiated controls (Leighton et al., 2013).

Lastly, as mentioned above, UVR is a known inducer of ROS, which are generated in skin tissue through photo-activation of endogenous photosynthesisors such as porphyrins, riboflavin and quinones (Marrot and Meunier, 2008). ROS include superoxide, hydrogen peroxide, singlet oxygen and hydroxyl radical, which have been shown to initiate

signalling pathways indicative of cell damage and apoptosis (Black and Lambert, 2001; Tyrrell, 1995), demonstrating an alarmin role for ROS following UV-B exposure.

Data from the above studies provide evidence that physiologically relevant doses of UV delivered both *in vivo* and *in vitro* stimulate the secretion and release of alarmins and pro-inflammatory mediators that contribute to a hyper-inflammatory state. Once in the extracellular space, alarmins work similarly to the exogenous pathogen associated molecular patterns (PAMPs) and together they fall under the broader category of damage-associated molecular patterns (DAMPs), leading to further exacerbations of the inflammatory response (Bianchi, 2007).

2.6 Alarmins contribute to inflammation through binding to pattern recognition receptors such as TLR4, leading to downstream release of pro-inflammatory products

Alarmins released during UV stress must alert the immune system of danger in order to promote repair, thereby initiating an inflammatory response through the activation of specific pattern recognition receptors (PRR) which are present on surrounding epidermal keratinocytes/melanocytes (Miller, 2008). Receptors from these families may also be found within dermal tissues on endothelial cells of the microvasculature, as well as on stromal cells, such as fibroblasts and adipocytes (Miller, 2008). Activation of PRRs can stimulate a multitude of responses, including cell differentiation, cell death, or secretion of anti- or pro-inflammatory mediators (Ojcius and Saïd-Sadier, 2012). The particular PRRs of interest in the pro-inflammatory pathway are the TLR family and nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs), both of which contribute to an enhanced inflammatory state.

TLRs are transmembrane glycoproteins that all contain two main signalling domains. Firstly there is an ectodomain of leucine-rich repeat (LRR) motifs that are able

to specifically recognise components on DAMPs (Akira et al., 2001). The second is a cytoplasmic domain, containing a toll/interleukin receptor (TIR) responsible for initiating several intracellular signalling cascades (Akira et al., 2001). These signalling cascades include activation of transcription factor nuclear factor (NF)-κB, which is a key factor in stimulating the expression of genes involved in immune responses such as cytokines, chemokines and co-stimulatory and adhesion molecules (Miller, 2008).

NLRs are another family of PRRs that can also recognise microbial peptides and intracellular alarmin signals, initiating host defence pathways via the NF-κB response and inflammatory caspases (Nasti and Timares, 2012). Activation of NLR stimulates the formation of the inflammasome, comprised of a sensor, an adaptor and a zymogen procaspase-1, and leads to caspase-1 activation. Caspase-1 is responsible for the maturation and release of IL-1β (Feldmeyer et al., 2007; Johnson et al., 2013). Induction of IL-1β expression and its release can subsequently induce the expression of additional inflammatory mediators, therefore further amplifying the inflammatory response (Nasti and Timares, 2012).

The direct link between UVR and activation of TLRs and NLRs is yet to be fully established, but reports indicate that both receptor families are likely contributors to UV-induced inflammatory processes (Nasti and Timares, 2012). Endogenous DAMP signals that bind to TLRs include HSP60, HSP70, gp96 (from the HSP90 family), fibrinogen, heparan sulfate, β-defensins, HMGB1 and mRNA which initiate signalling pathways and stimulate pro-inflammatory cytokine production through receptor ligation (Erridge, 2010; Miller et al., 2011; Tsan and Gao, 2004). Whereas, ATP, uric acid and ROS can initiate inflammation via activation of the inflammasome complex (Chen et al., 2013; Leighton et al., 2013; Martinon et al., 2006; Tschopp and Schroder, 2010). As discussed in the previous

section, many of these endogenous DAMP signals are alarmins released from UV-damaged skin cells.

2.7 Peripheral TLR4 activation can influence neuronal signalling and alter behaviour through immune-brain communication pathways

The evidence discussed so far has demonstrated that UVR induces an inflammatory response in the skin, through the release of endogenous alarmins. We have also discussed how inflammatory signalling plays a role in addiction, building the foundation for the hypothesis that an alarmin driven mechanism may contribute to the addictive-like behaviour observed in frequent tanners through TLR4. Therefore, it is important to now consider how peripheral TLR immune signalling may influence higher brain centres.

Currently, no direct evidence of a UV-alarmin-brain pathway exists. However, parallels can be drawn to the immune-to-brain communication pathways associated with the sickness response, in which peripheral TLR4 activation, contributes to behavioural adaptions following infection (Hines et al., 2013). The sickness response describes the set of profound behavioural changes that occur with illness, including increased anhedonia, malaise, loss of locomotion and anorexia. Sickness behaviours result from an independent neuroinflammatory state initiated by endogenous inflammatory mediators (Dantzer, 2004). Once thought to be unfortunate side effects of illness, this shift in behaviour is now recognised to be a highly adaptive and organised strategy of cytokine-dependent signalling cascades that promote homeostasis (Dantzer, 2001).

The brain monitors peripheral immune responses by several pathways that act in parallel. Firstly, there is a humoral pathway, in which peripheral TLRs in macrophage-like cells residing in the circumventricular organs and the choroid plexus respond to circulating DAMPs. As this area of the blood-brain-barrier is “leaky”, it enables direct increases in

neurokine signalling through volume diffusion (Saper and Breder, 1994). In a second, neural pathway, locally produced cytokines may activate primary afferent nerve endings, such as the vagus nerve, contributing to behavioural change via neural mechanisms (McCusker and Kelley, 2013; Van Dam et al., 1993). A third pathway, consists of cytokine transporters across endothelial cells at the blood-brain-barrier, allowing pro-inflammatory cytokines to gain access to the brain via saturable transport systems (Banks et al., 1991). A fourth pathway involves binding of inflammatory cells to adhesion molecules, such as ICAM (intracellular adhesion molecules) and VCAM (vascular adhesion molecules) on the endothelium of blood-brain barrier vessels, enabling cells to migrate through the tissue (Wong et al., 2007). Finally, peripheral endogenous signalling may communicate across the blood-brain-barrier through peripheral leukocytes as they roll along and adhere to cerebral vasculature (D'Mello et al., 2013). Engagement of these immune-to-brain communication pathways ultimately stimulates the "activation" of glial cells, which are the non-neuronal support cells of the central nervous system (CNS) responsible for the central production of inflammatory cytokines (Jacobsen et al., 2014; Laflamme and Rivest, 1999). As glia are anatomically co-localised at the tetrapartite synapse, a key site of interaction between astrocytes, microglia and neurons, they enable bidirectional communication between cells, facilitating "neuroimmune communication" (De Leo et al., 2006; Eyo and Wu, 2013). Therefore, glial cells have the ability to rapidly respond to perturbations in either peripheral or central homeostasis and directly influence neuronal activity, critical for regular physiological function as well as pathological states (Milligan and Watkins, 2009)

Cytokines in the brain can function as part of an integrated network of signal amplification inducing potent effects on neural pathways, ultimately directing behavioural output (Capuron and Miller, 2011). For example, it was demonstrated that an illness response stimulated by systemic administration of an endotoxin (lipopolysaccharide; LPS)

caused significant elevations of dopamine release in the NAc, despite decreased intracranial self-stimulation on ascending rate-intensity functions (Borowski et al., 1998). This study provides a specific example of a TLR4-mediated illness response affecting central dopamine release, a key neurotransmitter involved in reinforcing behaviour, within the NAc (Sulzer, 2011). Another study reported that peripheral administration of recombinant IL-1β, IL-2 and IL-6 contributed to alterations in norepinephrine, serotonin and dopamine activity within multiple brain regions that were cytokine specific (Zalcman et al., 1994). Hence, there is already good evidence to demonstrate that immune stimuli of diverse origins can modify behaviour, paving the way for UVR as an immune stimulus to cause adaptations in reward behaviours.

Each of these examples of immune-brain signalling provide a potential mechanism, by which UV-mediated alarmin signalling may influence neurotransmission and behavioural outcomes. Therefore, it is hypothesised that UV-induced alarmin activation of TLRs, either peripherally or centrally, directs neurotransmission and may drive an addictive-like behavioural response (Figure 2.1).

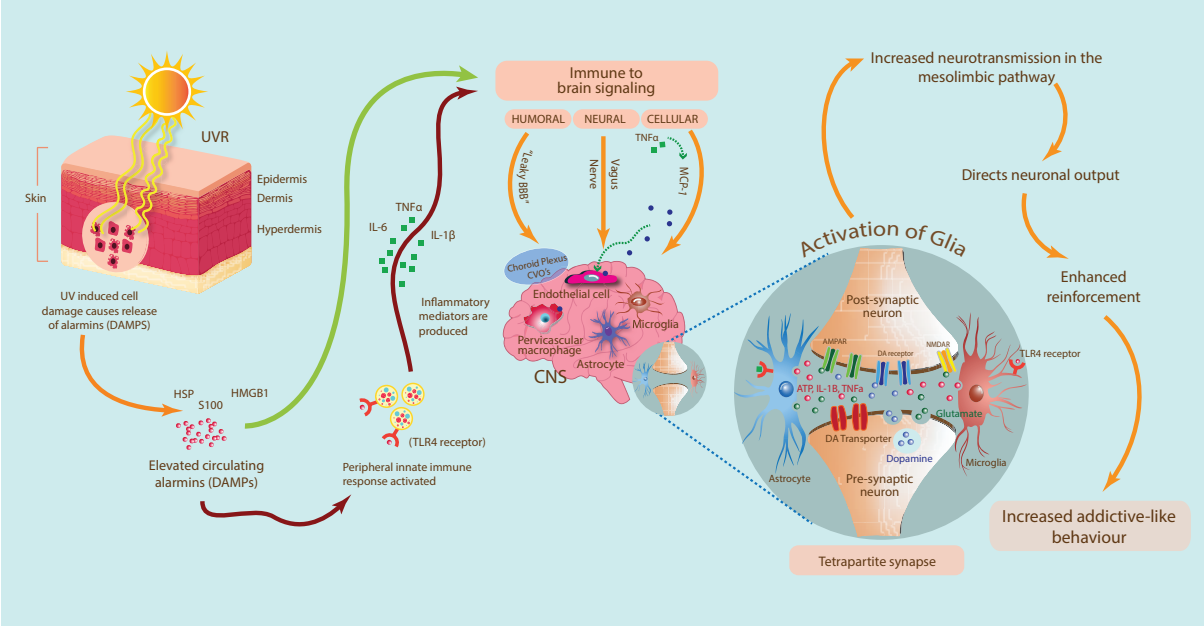


Figure 2.1 A schematic representation of the proposed immune-to-brain signalling pathways that may be induced by UVR. UVR damages skin cells, resulting in the production of "alarmins". Alarmins are DAMP signals that serve to alert the immune system of danger and are detected by immune competent cells, leading to stimulation of pro-inflammatory cytokines and chemokines. Based on identified pathways of peripheral to central communication, pro-inflammatory mediators released from peripheral macrophages and monocytes or alarmins could access the CNS via the following pathways: 1) Humoral pathway - Circulating DAMPs and pro-inflammatory cytokines may activate TLRs on the perivascular macrophages to access the brain through leaky regions of the blood-brain-barrier, such as the choroid plexus and circumventricular organs (CVO's). Within neuronal tissue, the activation of endothelial cells leads to the subsequent release of second messengers such as prostaglandins (PGE_2) and nitric oxide (NO). 2) Neural pathway – Peripheral pro-inflammatory cytokines may stimulate afferent nerve endings such as the vagus nerve, which are then relayed within the brain. 3) Cellular pathway - peripheral leukocytes can adhere to cerebral vasculature signalling across the blood-brain barrier. Pro-inflammatory mediators, namely TNF-α, contributes to the production of

monocyte chemoattractant protein-1 (MCP-1), which is principle for the recruitment of monocytes within the central nervous tissue. These immune-to-brain signalling pathways contribute to the activation of glial cells (astrocyte and microglia). Glia, together with the pre- and post-synaptic neurons, form the tetrapartite synapse, enabling bidirectional communication between cells. Activation of glia is characterised by higher concentrations of inflammatory mediators, creating a neuroinflammatory environment which impacts on the excitation of neurons (increased AMPA and NMDA receptors, increased glutamate in the synapse, downregulating of GABA). Increased inflammatory signalling at neuroanatomical sites associated with addiction, the mesolimbic dopamine pathway, could contribute to enhanced reinforcement increasing addictive-like behaviour**.**

2.8 We don't like being sick, so why do we "like" UV exposure?

Our understanding of the immune system is ever evolving, and it has become apparent that the one response fits all approach may not be true in every situation. Therefore, it is important to note that immune responses are context specific and will vary according to the stimulus (Graeber, 2010). Key factors, such as neuroanatomy and the heterogeneity of the immune system, significantly affect how a peripheral immune response can initiate a central response (Hutchinson and Watkins, 2014). This means that peripheral immune responses do not simply cause pan-glial reactivity but, through discrete and selective pathways, may lead to distinct behavioural phenotypes; such as eliciting an illness response in one situation and contributing to the development of addiction in another (Hutchinson and Watkins, 2014).

In the case of sickness behaviours, there is a significant insult to the peripheral immune system, which requires an adaptive response, affecting multiple behavioural (anhedonic, appetite, mood) and physiological (fever) outcomes (Liu et al., 2014). This

response is pro-inflammatory, and relies upon TLR activation of peripheral immune cells significantly increasing circulating inflammatory products, as the blood-brain barrier largely prevents endotoxins from readily entering the CNS (Quan and Banks, 2007). This anatomical barrier immediately limits direct glial activation, ensuring a slower, but more graded glial response. Conversely, recreational substances, such as opioids and alcohol, can readily penetrate the blood-brain barrier, activating glia directly (Stevens et al., 2013). Alarmins signals may also activate glia directly, as they can readily enter neuronal tissue through humoral pathways or they may be released within the CNS directly as a result of neuroinflammation (Bianchi, 2007). Direct access of alarmins to glia means that alarmins can function akin to neurotransmitters and therefore act at sub-inflammatory levels much lower in magnitude than an illness response (Hutchinson and Watkins, 2014).

Furthermore, the physiological responses to UV exposure are not mutually exclusive or occurring independently of one another (D'Orazio et al., 2013). The hypothesis presented here builds on the evidence that together neuroimmune signalling and opioid/dopamine signalling produce a heightened response, which may contribute to the addictive-like behaviour observed in certain individuals following UV exposure. Interestingly, recent work by Petrulli and colleagues (2017) demonstrated a synergistic effect between immune activity and dopamine stimulants (Petrulli et al., 2017). Intravenous administration of LPS contributed to elevated stimulant-induced dopamine concentrations in the striatum (detected with PET scan) of human subjects, when compared to those receiving placebo. Yet, despite elevated circulating inflammatory cytokines, the negative behavioural effects typically observed during an illness response, such as fatigue, were not present (Petrulli et al., 2017). These findings suggest that there may be a synergistic effect between stressors leading to immune system activation and increased dopamine signalling, with the presence of inflammation perhaps increasing addiction potential. Such a finding

could have significant implications for tanning addiction, as chronic UV exposure may lead to similar stressor-induced immune activation, and, consequently, increases in central dopamine signalling, although this hypothesis remains to be empirically tested.

It is also interesting to note that in instances of both illness response and drug abuse, the resulting inflammation would be much more transient than the inflammatory response initiated from UV-damage, and would be resolved, under normal conditions, when the stimulus was removed. Comparatively, the inflammatory response triggered by UV exposure would presumably be much lower, but with increased frequency, due to the ubiquitous nature of sunlight. This is of particular relevance in countries where the UV index is high, and people tan frequently. Constant UV exposure would provide low grade, everyday stimulation of cell stress signals; an effect that would be even further increased in frequent tanners. This could have significant ramifications for basal glial priming reactivity (linked to priming of heightened responses) and could contribute to the maladaptive addictive-like behaviours demonstrated by frequent tanners.

There is still a great deal unknown regarding the hypothesised link between UV exposure and behavioural changes. However, there are significant converging lines of evidence that can direct future research. The sun-tanning literature demonstrates that people who are exposed to UV, present with signs of withdrawal, signifying dependence (Harrington et al., 2011b; Warthan et al., 2005). The physiological process of tanning is a trigger for β-endorphin production, which has the capacity to activate neuronal signalling within key brain regions that have been associated with addiction (Fell et al., 2014; Volkow and McLellan, 2016). Given the new insights into neuroimmune modulation and the role of glia in facilitating dependence (Hutchinson et al., 2012), combined with the hypothesised UV-DAMP signal to the brain, the opportunity for a new neuroimmune contributor exists. Therefore, it is possible that UV induced alarmin signalling, which arises in the skin

following UV exposure, could be contributing to glia activation and play a part in the addiction-like behavioural phenotype observed following UV exposure.

2.9 Conclusion

Our hypothesis outlined in the current review posits that UV exposure causes behavioural modifications in those who tan frequently and that this behaviour is comparable to that which is seen in drug addiction. UVR activates components of the mesolimbic dopamine pathway, indicating that dopamine neurotransmission may be a key target for UV-mediated behavioural adaptions. UVR also leads to elevations in the opioid neuropeptide β-endorphin, suggesting involvement of opioid neurocircuitry systems as well.

To date, a direct mechanism by which UVR can influence neurotransmission to modify behaviour is still to be identified. However, recent advances in neuroimmunology point to a possible mechanism by which UV exposure could influence central neurokine signalling. A chronic alarmin-mediated process derived from repeated UV-initiated cellular damage is hypothesised to lead to TLR/NLR activation, causing the peripheral release of pro-inflammatory cytokines. These alarmins and pro-inflammatory cytokines could penetrate or translate the CNS, where they may cause activation of central TLRs and other inflammatory receptor systems, translating to a state of glial reactivity. This glial activation would then lead to enhanced opioid and dopamine signalling within the mesolimbic circuitry, facilitating addiction-like behaviours in frequent tanners. Further research is required to investigate the novel cutaneous-to-brain immune mediated hypothesis presented here, as a potential mechanism explaining the addictive-like behavioural adaptions observed with frequent tanning and UV exposure. If found to be clinically

applicable, these hypotheses also point to the need to expand the range of interventions and services available in the public health programs used to promote sun smart behaviours.

Thesis aims and hypotheses

The work contained in this thesis aimed to understand how UV light applied to the skin has the capacity to influence neuronal circuitry and affect neurological outcomes. Thus, this work investigates cutaneous responses to UVR and the potential skin-induced signalling molecules that communicate from peripheral to central tissue. Chapter 3 focuses on an immune mediated-mechanism, whereas Chapter 4 explores the contributions of vitamin D.

Chapter 3. Do UV-induced alarmin stress signals influence dopaminergic brain pathways and increase motivation?

Given the recent advances in the field of neuroimmunology demonstrating that heightened immune signalling has the capacity to modify behavioural outcomes (Hutchinson and Watkins, 2014; Narita et al., 2006), it is plausible that UV-induced inflammatory signalling could be a contributing mechanism for the increased UV-seeking phenotype observed in frequent tanners. Therefore, the aims of the first study were to:

1. Develop and optimise a novel apparatus using fibre optic technology for the safe and targeted exogenous delivery of UV light to the skin of Sprague Dawley rats (275-400g).
2. Determine the alarmin profile in the skin, blood, spinal cord and brain tissue following UVR exposure at different levels/intensities of exposure.
3. Characterise both the peripheral and central UV-induced immune profiles through the measurement of inflammatory markers from the blood, spinal cord and brain.
4. Examine dopamine-related behaviours and dopamine levels within the nucleus accumbens using operant conditioning boxes paired with microdialysis and immunohistochemistry.

As UVR is a potent inducer of cell damage, it was hypothesised that UV exposure will contribute to an immune response and the release of endogenous alarmins that will impact on dopamine neurocircuitry. Further, this will contribute to alterations in dopamine neuronal signalling that increase behavioural output in a test of motivational drive.

Chapter 4. Are the protective benefits of vitamin D in neurodegenerative disease dependent on route of administration? A systematic review.

UV light is also responsible for the cutaneous production of vitamin D and this may be another pathway connecting peripheral UV with central changes. Several studies indicate that sun exposure mitigates neurological disease and is protective against the risk of neurodegeneration, an effect that has largely been attributed to synthesis of vitamin D (Annweiler et al., 2011; Duan et al., 2014; Landel et al., 2016; Munger et al., 2006; Shen and Ji, 2015). To investigate a role for vitamin D in neurodegenerative disease a systematic review was completed that aimed to:

1. Determine if neuroprotective benefits from vitamin D in neurogenerative disease are dependent on route of administration (exogenous or endogenous) by reviewing published literature available on PubMed, Embase and PsycInfo.

It was hypothesised that if vitamin D was the sole mediator of UV related protective benefits, then synthetic vitamin D supplementation would either improve disease outcomes or reduce disease risk.

Chapter 3. Do UV-induced alarmin stress signals influence dopaminergic brain pathways and increase motivation?

This chapter describes the proposed experimental work designed to investigate if UVR influences dopaminergic systems within the brain to increase motivated behaviour; and if this is driven, at least in part, by alarmin signalling arising from UV-damaged skin cells. The chapter is written as a research proposal and includes a brief project summary and discussion of the two main research questions. The project aims, and hypotheses are then presented which are followed by a detailed outline of the methodology and analysis techniques that have been carefully considered and planned. This chapter is written in this format as the work was not completed due to unforeseen circumstances, which significantly reduced my capability to undertake the research. Progress of the work that had commenced can be viewed in the Appendices.

3.1 Brief summary

This project will examine if alarmin cell stress signalling arising from UV damaged skin cells contributes to increased reinforcement or motivation, through the analysis of operant choice behavioural paradigms and dopamine levels within the nucleus accumbens (NAc).

The proposed study design will use a dose-response-like application of graded UVR concentration applied directly to the skin of the scapular region of Sprague Dawley rats. The first aim is to investigate whether a correlation exists between cutaneous alarmin production and behavioural modifications. Tissue analysis for levels of alarmins, secondary markers of inflammation and dopamine will be performed on the skin, blood, spinal cord and brain using a combination of immunohistochemistry, molecular pathology and high-

performance protein liquid chromatography (HPLC). An acute effect will be recorded following a single UV exposure, whereas a chronic effect will be measured following daily exposure for a period of 28 days. This study will use the well-validated concurrent choice procedure (Randall et al., 2012) to investigate how UVR impacts effort-related choice behaviour. Additionally, to further consolidate findings, a second microdialysis study is proposed to measure the levels of dopamine in cerebrospinal fluid from the NAc within a freely moving animal.

3.2 Research questions

3.2.1 Is UVR associated with increased motivational output and the continued engagement in operant lever pressing? Does this correlate with elevated production of alarmins and immune activation?

Rationale: People who tan frequently can exhibit signs of psychological and physical dependence suggesting that UVR has a reinforcing effect associated with increased motivation for sun-seeking behaviour (Feldman et al., 2004; Fell et al., 2014; Harrington et al., 2011a). As such, tanning may present as a form of behavioural addiction with similar impacts on neurocircuitry to other types of addiction, particularly affecting the mesolimbic dopamine pathway (American Psychiatric Association, 2013; Aubert et al., 2016; Kourosh et al., 2010; Nolan and Feldman, 2009). The dopamine neurotransmitter is heavily associated with the development of addiction, due to its involvement in the neurocircuitry of motivation and associative learning (Salamone and Correa, 2012). Dopamine is known to increase the motivation to seek rewarding stimuli, or the "wanting" component of addiction with dopaminergic drive increasing as addiction develops (Berridge et al., 2009). Increases in dopamine efflux have been observed in frequent tanners following exposure to UVR (Aubert et al., 2016), thus it is reasonable to hypothesise that UVR stimulation of

dopaminergic pathways may play a critical role in the behavioural modification observed with frequent tanning.

To date, the physiological mechanisms by which peripheral UVR could be driving behavioural change via secondary neuronal signalling have yet to be defined. However, recent developments in addiction research and the discovery of an immune contribution to addiction pathophysiology, open the possibility for previously unidentified signalling pathways (Hutchinson and Watkins, 2014). Solid evidence shows that vulnerability to addiction is increased during a pro-inflammatory state and that inflammatory mediators change the pharmacodynamics of illicit substances (Hutchinson et al., 2008; Narita et al., 2006). As UVR has the capacity to initiate inflammatory cascades and the release of endogenous alarmins (Byrne et al., 2011; Johnson et al., 2013; Takai et al., 2011; Yoshizumi et al., 2008), this may be one potential pathway by which UVR could affect central circuits and alter behavioural outcomes. Therefore, this project proposes to investigate the effects of cutaneous UVR on alarmin expression (in the skin, blood, spinal cord and brain) and if this correlates with increased performance in a test of motivated behaviour using the progressive ratio/concurrent choice procedure (Randall et al., 2012).

The progressive ratio/concurrent choice procedure is a well validated behavioural test capable of detecting alterations in dopamine expression, particularly within the NAc (Randall et al., 2012; Salamone et al., 1997). In this task, experimental animals are given the choice to engage in lever pressing (that is continually demanding more effort by increasing the number of required lever presses) for a highly desirable sugar pellet or to consume the concurrently available, less palatable standard chow (Randall et al., 2012). Administration of dopamine antagonists produces a dramatic shift in the choice behaviour of animals resulting in decreased lever pressing for the preferred chow and considerable increases in consumption of the freely accessible chow (Koch et al., 2000; Salamone et al.,

2001). Whereas, elevated levels of dopamine within the NAc, such as through administration of amphetamines or knock down of dopamine transporters, contributes to decreased performance in motivation under a progressive ratio schedule of reinforcement (Zhang et al., 2003). Consequently, using this behavioural paradigm, sensitive to dopamine alterations in conjunction with UVR exposure, will shed light on whether UVR affects neurocircuits in a manner similar to other dopamine manipulators. This will test the hypothesis that UV exposure will contribute to the release of endogenous alarmins and this will have a central effect increasing behavioural output during a test of motivated choice behaviour.

3.2.2 Is UV exposure associated with increased dopamine within the nucleus accumbens in a dose dependent manner?

Rationale: In addition to behavioural modifications attributed to UVR, it is also important to consider the neurotransmitter signalling underlying any adaptations to motivated behaviour and provide evidence of such change. As mentioned in the previous section, mesolimbic dopamine is the most likely candidate as it plays a key role in several behavioural functions related to motivation (Bromberg-Martin et al., 2010). Moreover, the mesolimbic dopaminergic pathway is also heavily associated with the development of addiction with dopamine efflux implicated in drug/alcohol abuse as well as a number of behaviours that can become addictions (Avena et al., 2009; Balfour et al., 2004; Di Chiara and Imperato, 1988). In addition, mesolimbic activation and dopamine increases have already been recorded in frequent tanners following tanning sessions. Firstly, a study using SPECT imaging to measure rCBF during a session with either UVR or sham UVR in frequent tanners, demonstrated that during the UVR session, tanners had a significant increase in rCBF in the left striatum (Harrington et al., 2011a). Another group reported dopamine efflux occurred in frequent tanners, but not in infrequent tanners after UVR

administration. This effect that was diminished when using sham UVR light, in which UV wavelengths were blocked (Aubert et al., 2016), providing further support that dopamine could be the key neurotransmitter involved in modifying behaviour following prolonged UV exposure. Dopamine neurotransmission is also known to be sensitive to inflammatory signalling and therefore may respond to increases in circulating alarmins induced by UVR. Increased efflux of *in vivo* dopamine from the NAc was observed following peripheral injections of the inflammatory stimulant lipopolysaccharide (LPS) (Borowski et al., 1998). This suggested that in addition to illness-induced behavioural modifications, inflammatory stimuli can also provoke alterations in neurotransmitter release along the mesolimbic pathway. Therefore, this project will investigate if UV light can influence dopamine signalling, testing the hypotheses that UVR exposure will increase dopamine levels within the NAc in a dose dependent manner.

3.3 Aims

1. Develop and optimise a novel apparatus using fibre optic technology for the safe and targeted exogenous delivery of UV light to the skin of Sprague Dawley rats (275-400g).
2. Characterise the alarmin immune response in skin, blood, spinal cord and brain tissue following exposure to different durations of UV light.
3. Characterise the peripheral and central UV-induced immune profiles by measuring inflammatory markers from the blood, spinal cord and brain.
4. Examine dopamine related behaviours and dopamine levels within the NAc of Sprague Dawley rats by monitoring performance in motivated behavioural task (concurrent choice behaviour) paired with analysis of microdialysis and immunohistochemistry.

3.4 Hypotheses

1. UVR exposure will contribute to an immune response and the release of endogenous alarmins from irradiated skin cells.
2. This UV-induced alarmin signalling will contribute to central alterations effecting dopamine levels within the NAc. UV-triggered dopamine efflux will occur in a dose-dependent manner.
3. Any increases in dopamine will affect behavioural output and contribute to increased performance in the progressive ratio/concurrent choice behavioural paradigm used to test motivated behaviours.

3.5 Experiment 1: Examine the effect of daily ultraviolet exposure on motivational drive and determine ultraviolet induced alterations in the alarmin response in rodents

Overview: The aim of this study is to investigate the alarmin response generated from graded concentrations of chronic dermal UVR and if this correlates to a change in dopaminergic-driven behaviour. This will allow us to determine whether UVR alters neuronal processing causing a change in behaviour. The behavioural test used for this study will follow the protocol published by Randall et. al 2012, using a progressive ratio/concurrent choice paradigm, in which the animal can either lever press (with increasing effort) for a desired high carbohydrate pellet or consume freely accessible standard chow (Randall et al., 2012).

Rats (n =50; 10/group) will be trained on the concurrent progressive/chow feeding procedure as described in section 3.7.3 below. The sample size (n=10/group) has been selected from a power analysis using a standardised effect size of 1.4 (signal/noise ratio),

believed to provide the study with sufficient power (80%) for statistical analysis, as there is no preliminary data to guide group size estimates.

UVR exposure will be administered using a between-groups design, with each rat receiving only one exposure condition. Rats will be randomly assigned to receive either 0, 0.5, 1, 2 or 4 kJ/m^2 UVR delivered via a newly designed UV apparatus, that will guide UV light directly onto the skin (described in section 3.7.2). Immediately following UVR exposure, rats will be placed within an operant conditioning chamber where they will begin the progressive ratio concurrent choice feeding session, as a test of motivated behaviour. Behavioural measures to be recorded include the total number of lever presses/pellets received within the 30-minute period and the amount of freely accessible chow consumed. UVR exposure and choice behavioural testing will be performed daily for a period of 28 days. At completion of the experiment animals will be humanely sacrificed and tissue collected for further analysis. Half of the animals from each group (n=5) will be prepared for immunohistochemistry (IHC) and serial sections from the brain/spinal cord will be stained using specific antibodies to measure the presence of alarmins (HMGB1, IL-1β, IL-33) and immune activation (CD68, CD3, RAGE and TLR4 receptors) within key regions of interest. The remaining animals (n=5) will be prepared for molecular pathology and protein quantification using the western blot technique and enzyme-linked immunosorbent assay (ELISA) for markers of protein expression and immune activation (based on results from IHC).

3.6 Experiment 2: Determine the effect of ultraviolet exposure on the levels of dopamine within the nucleus accumbens

Overview: The aim of this study is to investigate the effects of graded concentrations of chronic UV light on dopamine levels within the NAc, using microdialysis sampling to

detect changes in neurotransmitter levels. This will shed light on whether UVR influences dopaminergic transmission within the NAc.

Rats (n =50; 10/group) will be implanted (unilaterally) with a 10-mm microdialysis guide cannula (described in section 3.7.8) to allow sampling of cerebrospinal fluid (CSF) from the NAc to measure dopamine concentration. This group size has been selected based on previous microdialysis experiments and is expected to provide sufficient statistical power (see (Collins-Praino et al., 2012; Ishiwari et al., 2004).

UVR exposure will follow the same protocol as Experiment 1, using a between groups design and random assignment of either 0, 0.5, 1, 2 or 4 kJ/m^2 UVR, to be administered with the UV apparatus. Rats will be exposed to UVR daily for 28 days but microdialysis samples of CSF will only be collected on days 1, 7, 14 and 28. On sampling days, animals will have a concentric dialysis probe inserted through the guide cannula. Artificial CFS (aCSF) will then be continually perfused through the microdialysis tubing at a flow rate of of 2.0 μL/min. Prior to UVR exposure, flowing CSF will be sampled every 30-minutes until seven baseline samples are collected. Animals will then be fitted with the UV apparatus and receive their daily dose of UVR. During which time, animals will remain in the microdialysis chamber and CSF sampling will continue for a 6-hour period with samples collected every 30-minutes. CFS samples will be analysed for dopamine content using reverse-phase HPLC with electrochemical detection. Food and water will be available *ad libitum* within the microdialysis chamber.

3.7 Proposed methodology

This section describes the general procedures to be utilised for the preceding experiments. However, as this is a new research design at the University of Adelaide, pilot experiments will be necessary to determine key experimental parameters.

3.7.1 Animals

Male Harlan Sprague Dawley rats weighing between 275-400g will be used for all experiments. Sprague Dawley rats have been selected as they have been previously used by our lab to explore behavioural consequences of inflammatory signalling. This strain is also regularly used in behavioural studies and in addiction research, therefore ensuring the results of this study will be comparable to other published data within the field (Hutchinson et al., 2012; Randall et al., 2012). Rats will be housed in a temperature (23±3°C) regulated facility and subject to a 12 h light/ 12 h dark cycle. Animals will have *ad libitum* access to chow and water in the home cage unless assigned to the progressive ratio behavioural groups in which animals will be food deprived to 85% of their free feeding body weight for initial training purposes. Rats will be fed supplemented chow throughout the behavioural study, with *ad libitum* access to water in the home cage. Hair from the scapular region will be continually removed using an electric shaver throughout the study period to allow for direct UVR exposure on the skin. All experimental procedures will be performed in accordance with the National Health and Medical Research Council Australian Code for the care and use for scientific purposes (8th edition, 2013) and the University of Adelaide Animal Ethics Guidelines, and approved by the University of Adelaide Ethics Committee.

3.7.2 Ultraviolet light apparatus

To best investigate the effects of different UV light concentrations, a novel UV light apparatus will be employed to allow for the safe and targeted delivery of UVR. This design guides light directly onto a small portion of shaved skin, serving to eliminate unnecessary exposure to sensitive areas of the animal (ear, paws and eyes) and to ensure the precise delivery of exact quantities of UVR by eliminating external variables that might affect the amount UVR received. Furthermore, the advanced fibre optic technology available also has

the capacity to obtain a reflection-absorption spectrum, providing information of irradiance projected back off the skin which main serve to provide additional insights on skin related changes following exposure to UVR.

The design consists of a lightweight titanium dispersing cone that is to be worn by the animal (embedded within a fabric vest) projecting light on to the shaved scapular region. The dispersing cone is connected with fibre optic cables to a plasma laser-driven light source (Eq-99, Energetiq Technology Inc., Wilmington, MA, United States), emitting wavelengths of light that are comparable to those emitted from sun as recorded at sea level. However, as this apparatus employs a new method for delivery of UV light, pilot experiments will be needed to identify the appropriate concentration of UVR to in order to address the specific research questions. The proposed intensities of UV light have been based on peer-reviewed literature and include 0, 0.5, 1, 2 and 4 kJ/m^2 of UVR. These intensities have been selected to create a dose-response like regime and to represent a range from minimal to mild sunburn (Grimbaldeston et al., 2007; Yoshizumi et al., 2008).

Each exposure session will last the duration of the 30-minutes, however, the manipulation of UVR intensity will be achieved by using a UV filter (blocking all wavelengths below 400nm) to create "sham" UVR. The length of UVR exposure needed to fulfil each intensity, determined in minutes, will be calculated and the UV filter applied for the remainder of the 30-minutes session. The reflection-absorption measurement will be recorded daily prior to removal of the UVR filter at the beginning of the exposure session and then again once UVR exposure has ceased. This recording will be used to detect a potential shift in the amount of light that is absorbed/reflected by skin tissue. All exposure will take place inside an operant conditioning chamber, with the optic fibre cable extending from the top of the box. The animal will be able to move freely inside the chamber.

3.7.3 Behavioural testing

UVR-induced changes to motivated behaviour will be assessed using a progressive ratio/concurrent choice protocol. In this behavioural test, animals will have the choice to lever press for highly derisible sugar pellets or to eat the freely accessible standard rodent chow. However, the number of responses needed to release a sugar pellet are progressively increased throughout the test, thus continually more effort is demanded before a sugar pellet can be obtained providing a measurable outcome of motivated behaviour (Randall et al., 2012; Yohn et al., 2015). This test will be conducted daily within an operant conditioning chamber (28x23x23 cm^3; Med Associates Inc; Fairfax, VT, United States) and run for the duration of 30-minutes.

Initially, rats will be trained to lever press using a continuous fixed ratio (FR) reinforcement schedule for approximately one week, and then, will be shifted to the progressive schedule used by (Randall et al., 2012). In brief, the FR schedule will gradually be increased from FR1-FR5 (FR1- one lever press, one sugar pellet, FR5-five lever presses, one sugar pellet) across the training week. Once the animal has associated lever pressing with the delivery of a sugar pellet the progressive schedule will be introduced. Commencing with FR1, every time 15 reinforcements are obtained, the number lever presses needed will be increased by one additional response (FR1x15, FR2x15, FR3x15…). If a ratio is not completed within 2 minutes, a "time-out" feature will deactivate the response lever for the remainder of the session. Upon reaching a stable baseline of responses (approx. 4 weeks), chow will be introduced. Weighed amounts of standard rodent chow will be concurrently available on the floor of the chamber, the animal will now have the choice of eating freely available chow or to engage in lever pressing for sugar pellets, therefore testing motivational drive. Chow intake will be determined by weighing any remaining food (including spillage). Rats will be trained on the on the progressive schedule

for an additional 4 weeks, after which daily UVR exposure will commence in conjunction with operant testing.

3.7.4 Tissue collection

Prior to UVR exposure, animals will be anesthetised to perform a tail-vein bleed from which plasma will isolated and used to determine baseline levels of circulating alarmins (such as HMGB1, IL-1β, IL-33) and vitamin D. At cessation of behavioural testing animals will be humanely sacrificed for tissue collection including skin, blood, spinal cord and brain.

Firstly, skin samples will be acquired from the irradiated site and a proximal non-irradiated site using a 4mm punch-tool. Half of the samples from each location will be post-fixed in 10% neutral-buffered formalin (overnight at 4°C) in preparation for histological analysis. This will include routine hematoxylin and eosin (H&E) staining to detect UVR induced changes to skin integrity, cell type and cell number. Immunohistochemistry will also be performed using antibody staining to detect markers of immune activation. The primary antibodies of interest include HMGB1, IL-1β and IL-33, as these have been categorised as alarmins and have been shown by other research groups to alter their expression following UVR induced damage to keratinocytes (Byrne et al., 2011; Johnson et al., 2013; Yoshizumi et al., 2008). The remaining tissue will be snap frozen in liquid nitrogen to be stored for protein quantification and western blot analysis. Next, blood samples taken directly from cardiac tissue will be collected in EDTA tubes and the plasma used to measure potential UVR induced changes to both alarmin and vitamin D concentrations. Lastly, following skin and blood collection, half of the animals from each group will undergo transcardial perfusion with 10% neutral-buffered formalin, fixing the tissue for histological analysis and immunohistochemistry. The brain and spinal cord will

be analysed for the presence of alarmins (HMGB1, IL-1β, IL-33) and immune activation (CD68, CD3, RAGE and TLR4 receptors). The remaining animals will be perfused with 0.9% saline, to remove tissue of blood, and then the brain and spinal cord will be removed and snap frozen in liquid nitrogen. Brain and spinal tissue will undergo western blot, ELISA and multiplex analysis for protein expression of range of inflammatory markers including HMGB1, IL-1β, IL-33 and TLR4 and RAGE receptors. The data obtained from cutaneous and blood samples will be analysed first and will help determine the alarmin markers to be examined centrally.

3.7.5 Histological analysis

In preparation for H&E histological analysis of treatment groups, fixed samples must undergo tissue processing during which water is removed and replaced with paraffin wax, enabling thin sections to be cut and viewed microscopically. Firstly, tissue samples will be placed in processing cassettes and then systematically transferred through a series of baths gradually increasing in ethanol content (70-100%), dehydrating the tissue of water. Samples will then be submerged in the clearing agent xylene, necessary to remove the ethanol from the tissue to allow the paraffin wax to infiltrate the tissue. After processing is complete, samples will be externally embedded in wax with sections orientated so all layers of the skin can be identified. Once the wax has solidified, serial sections will be cut using a rotary microtome (4μm) and mounted onto albumin-coated slides, followed by routine H&E staining. In brief, sections will be dewaxed with xylene and rehydrated through graded ethanol. Tissue sections will then be stained using Harris Hematoxylin (three minutes) before being placed in 0.5% ammonia (one minute) to enable bluing. Finally, sections will be stained with eosin (four minutes) and prepared for cover slipping, progressing through ethanol and xylene once again. After which, cover slips will be mounted, and the slides scanned with a NanoZoomer (Hamamatsum Photonics, Shizuoka

Pref., Japan). Tissue analysis will be performed on scanned images using NanoZoomer Digital Pathology software view.2 (Histalim; Montpellier, France) and ImageJ software (Schneider et al., 2012).

3.7.6 Immunohistochemistry

Immunohistochemistry (IHC) will be performed to determine the presence of alarmins (HMGB1, IL-1β, IL-33) and immune activation (CD68, CD3, RAGE and TLR4 receptors) using antibody staining. Each antibody will have a specific IHC protocol, recommended by the manufacturer, which will be used as a first point of reference. Although protocols may vary slightly, a general indirect IHC procedure using avidin is as follows:

Prepared tissue samples that have been fixed in in formalin and embedded within paraffin wax will be cut using a microtome (4μm) and mounted onto SuperFrost® glass microscope slides (Menzel-Glaäser; Braunschweig, Germany). Following dewaxing and rehydration steps, antigen retrieval will then be completed, necessary to break the amino cross-links that form during fixation to "unmask" the antigenic site of interest. This is commonly performed using heat treatment (such as within a microwave or pressure cooker) and will need to be optimised for each antibody. Generally, slides are submerged in a buffer solution (citrate, EDTA or Tris-EDTA) and then maintained at boiling temperatures for a set period of time (e.g 10 minutes). Slides must then be cooled to room temperature and endogenous peroxidase activity blocked using a 3% solution of hydrogen peroxide/ methanol. Further blocking of non-specific staining is required to reduced background interreference which can be achieved with the use of a blocking buffer, such as 10% normal horse serum (NHS). After the blocking stages are complete, slides are then incubated with the primary antibody, diluted in blocking buffer, within a humid chamber (typically

overnight). Incubation with the corresponding biotinylated secondary antibody is then applied followed by avidin horseradish peroxidase solution, which will further amplify the signal. Staining is then developed using 3,3' Diaminobenzidine (DAB) and slides are counterstained with hematoxylin and prepared to have coverslips applied. Once the coverslip mounts have dried, the slides can then be scanned using the NanoZoomer (Hamamatsu Photonics, Shizuoka Pref., Japan) and images analysed with NanoZoomer Digital Pathology software view.2 (Histalim; Montpellier, France) and ImageJ software (Schneider et al., 2012).

3.7.7 Molecular pathology

3.7.7.1 Tissue processing

In preparation for molecular pathology, skin tissue will be frozen in liquid nitrogen and crushed to a fine powder using a mortar and pestle and then sonicated with RIPA (radioimmunoprecipitation assay) buffer. Soft tissue samples will be homogenised and sonicated using RIPA, without the need for prior crushing. Homogenised samples will then be separated using a centrifuge set at 4°C/14,000 RPM and the supernatant collected. Total protein concentration will be calculated using a Pierce™ BCA protein assay (ThermoFisher Scientific; Waltham, MA, USA) at 750nm absorbance.

3.7.7.2 Western blot

The western blot will be used to detect and analyse modified protein quantities between treatment groups (determined from IHC), modelling techniques that have been previously published by our lab (Arulsamy et al., 2018). Briefly, gel electrophoresis will be performed using Bolt 4-12% Bis-Tris Plus gels (ThermoFisher Scientific; Waltham, MA, USA), loading 50ug of protein per well. Gels are then run at 150V for between 30-45 minutes (determined by the molecular weight of the protein of interest) and then to a PVDF

(polyvinylidene difluoride) membrane using the iBlot 2 Dry Blotting System (ThermoFisher Scientific; Waltham, MA, USA). Next, the membranes are repeatedly washed (3x5 minutes) using tris-buffered saline with tween 20 (TBST). Before progressing, the membranes will be submerged in Ponceau S red solution (Sigma-Aldrich; NSW, Australia) for five minutes, so that protein lanes can be visualised. All excess Ponceau S will be removed by rinsing the membrane with distilled water. Antibody staining will then be performed with primary and secondary antibodies in 1X iBind solution using the iBind Western System (ThermoFisher Scientific; Waltham, MA, USA). The specific antibodies selected will be based on results from IHC analysis but are expected to include markers for HMBG1 IL-1β, IL-33 and TLR4 and RAGE receptors. Imaging of western blots will take place using an Odyssey Infrared Imaging System (model 9120; software version 3.0.21) (LI-COR Inc; Lincoln, NE, United States) at a resolution of 169µm. Analysis will then be performed using ImageJ software (Schneider et al., 2012).

3.7.7.3 Enzyme-linked immunosorbent assay (ELISA)

UV-induced changes to cytokines, chemokines and other soluble biomarkers of interest will also be measured through the use of ELISA kits. The specific biomolecules to be measured with an ELISA have yet to be determined, however prior analysis of IHC and western blot will guide selection. Once identified, the appropriate ELISA kits will be utilised, following the manufacturer's specifications. A standard ELISA protocol, as presented on the ThermoFisher Scientific website, is as follows:

Prepared standard and tissue samples (50-100µL) will be added to a pre-coated 96-well plate and left to incubate at room temperature for a period of two hours. Specific biotinylated primary antibodies are then applied to each well, incubating for 60-minutes. Next, streptavidin horseradish peroxidase conjugate (100µL), which will bind to the biotin on the primary antibody, is added with an incubation period of 30-minutes. So far, in

between each of these preceding steps, the incubating solution must be adequately decanted and the plate washed, either using a squirt water bottle or plate washer. In the final incubating stage, a chromogenic colouring solution (100µL) is added to visualise substrates and must develop in the dark for 30-minutes. Lastly, a stop solution (100µL) is applied, preventing further colour development and absorbance can be read using a plate reader. Experimental data is then calibrated against the standard curve corresponding to each protein of interest.

3.7.8 Microdialysis and high-performance protein liquid chromatography (HPLC)

To investigate the effects of UVR on real-time levels of dopamine within the NAc, samples of CSF will be collected through microdialysis and screened using HPLC. Which will provide robust recordings of dopamine activity during UVR exposure to give a clear overview of UVR induced alterations to the dopaminergic system.

To facilitate sample collection, a microdialysis probe will be surgically implanted following well validated protocols (Collins-Praino et al., 2012; Ishiwari et al., 2004). Firstly, animals will have a 10mm guide cannular (Bioanalytical Systems Inc; Lafayette, IN, United States) unilaterally inserted into the NAc. To ensure correct placement of the cannula, a stereotaxic apparatus will be used with the upper incisor bar set at 5.0mm above the interaural line. The NAc can be located using the following coordinates: 8 mm anterior from bregma, 1.8 mm lateral from the midline, 6.8 mm ventral from the skull surface. Guide cannulae are then secured to the skull with stainless steel screws and polycarboxylate cement. The cannula will be counterbalanced and its integrity will be maintained by inserting a stylet in-between probe insertion. Animals will be allowed sufficient recovering time following cannula insertion.

On sample collection days, the stylet will be removed and a concentric dialysis probe (2.0 mm active surface; Bioanalytical Systems Inc; Lafayette, IN, United States) inserted through the guide cannula of each animal. The tip of the probe will extend 2.0 mm beyond the cannula with the active surface placed within the NAc. Artificial cerebrospinal fluid (aCSF; 147.2 mM NaCl, 2.4 mM CaCl2, 4.0 mM KCl) will then be continually perfused through connecting polyethylene tubing (flowrate of 2.0µL/min) with dialysis samples collected every 30-minutes. Seven baseline samples will be collected initially, prior to UVR exposure, to establish baseline levels of dopamine. UVR exposure will then be administered (0.5-4 kJ/m^2) and samples will continue to be collected every 30-minutes for up to six hours. Samples will be frozen, until analysis for dopamine content using reverse-phase HPLC with electrochemical detection (ESA Inc.; Chelmsford, MA, United States), following the methods developed by (Phillips and Cox, 1997).

3.8 Significance

The high levels of UVR in many countries across the globe are largely unavoidable, with almost everyone having some level of exposure on a daily basis. Increases in migration have also seen a rise in people dwelling in in environments which they are not genetically suited for and there has been a dramatic shift in clothing and lifestyle habits, greatly increasing the potential for harm attributed to UVR (Diffey, 2003; Leary and Jones, 1993; Jablonski and Chaplin, 2017). While there are benefits associated with sun and UVR exposure for vitamin D synthesis, there are numerous people receiving substantial quantities of UVR, beyond the necessary daily requirements (Diffey, 2003; Leary and Jones, 1993). Consequently, this dramatic increase in sun exposure enhances the known risks of UVR (e.g. skin cancer, cataracts, immune deficiency) but may also bring about new

UVR associated pathologies (e.g. behavioural addiction), with major consequences for public health.

Already, strong evidence suggests that UVR can act as a reinforcing stimulus, increasing sun-seeking behaviour in frequent tanners (Iacopetta et al., 2018). Yet, more research is needed to develop concepts further. Given that dopamine neurotransmission is a key target in addiction development, dopamine signalling along the mesolimbic pathway is a likely candidate for UV-mediated behavioural adaptions. By pairing sophisticated UVR administration technology with a test of motivated behaviour (sensitive to dopamine) and microdialysis sampling to monitor dopamine levels (within the NAc), the proposed research project will provide robust evidence to determine if UVR alters mesolimbic dopamine neurotransmission. This may provide new insights on the role of dopamine in the reinforcing properties of UVR. Furthermore, taking into consideration that neuroimmune modulation is also associated with the development of addiction, this project will look at the possible interaction between UV-induced inflammatory signalling and dopamine neurotransmission. The recent advances in addiction research have verified that inflammatory signalling can increase vulnerability to addiction through glial cell modulation. Therefore, UV-induced alarmin signalling arising from UV damaged skin cells may be the driving force behind the addictive-like behavioural adaptions observed with frequent exposure to UVR. By monitoring production of alarmin signalling at the site of contact, in the circulation, and within central tissue, it will be possible to determine if peripheral UV-induced inflammatory signals are relayed to the brain. This may shed light on a possible mechanism by which UVR can influence behavioural outcomes. If there is a correlation between immune activation and dopaminergic modification this could have further ramifications for understanding how the immune system contributes to addictive and motivational behaviours. And, if found to be clinically applicable, this work may

translate to the public health domain highlighting the need for broader interventions and services that promote sun-smart behaviour.

Overall, UVR-related research is of great significance to the population at large due to the global presence of UVR within the environment and the potential for harm associated with overexposure/avoidance.

Statement of Authorship

Title of Paper	Are the protective benefits of vitamin D in neurodegenerative disease dependent on route of administration? A systematic review.
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Iacopetta, K., et al. (2018). "Are the protective benefits of vitamin D in neurodegenerative disease dependent on route of administration? A systematic review." Nutr Neurosci: 1-30.

Principal Author

Name of Principal Author (Candidate)	Krystal Iacopetta		
Contribution to the Paper	Manuscript conception and design, reviewed all papers cited in the manuscript, wrote manuscript, designed figures and acted as corresponding author.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	18/12/2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Lyndsey E. Collins-Praino		
Contribution to the Paper	Supervisor, assisted with conception, preparation and editing of manuscript.		
Signature		Date	18/12/2018

Name of Co-Author	Femke T.A Buisman-Pijlman		
Contribution to the Paper	Supervisor, assisted with preparation and editing of manuscript.		
Signature		Date	18/12/2018

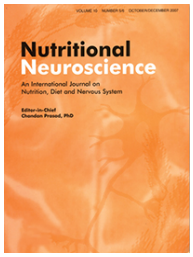
Name of Co-Author	Jiajun Liu		
Contribution to the Paper	Assisted with analysis of linguistic characteristics of Medline data and designed figures.		
Signature		Date	18/12/2018

Name of Co-Author	Amanda D. Hutchinson		
Contribution to the Paper	Assisted with conception, preparation and editing of manuscript.		
Signature		Date	18/12/2018
Name of Co-Author	Mark R. Hutchinson		
Contribution to the Paper	Supervisor, assisted with conception, preparation and editing of manuscript.		
Signature		Date	18/12/2018

<u>Chapter 4.</u> Are the protective benefits of vitamin D in neurodegenerative disease dependent on route of administration? A systematic review

This chapter has been peer-reviewed and formally published in a peer-reviewed journal [Iacopetta K et al. (2018) *Nutritional Neuroscience*].

In conjunction with inflammation and cell damage, skin exposure to UVR, at the same damage-inflicting wavelengths, is necessary for cutaneous production of vitamin D. For this reason, vitamin D and UV are virtually inseparable and vitamin D is closely imbedded with UV-related research. Moreover, it has been suggested that sun exposure attenuates several neurological diseases through the vitamin D pathway. This chapter presents a systematic review of the literature to address whether the presumed neuroprotective benefits attributed to vitamin D in neurological disease are equivalent concerning endogenous or exogenous derived vitamin D, teasing apart UVR and vitamin D in this context.



Taylor & Francis
Taylor & Francis Group

Nutritional Neuroscience
An International Journal on Nutrition, Diet and Nervous System

ISSN: 1028-415X (Print) 1476-8305 (Online) Journal homepage: http://www.tandfonline.com/loi/ynns20

Are the protective benefits of vitamin D in neurodegenerative disease dependent on route of administration? A systematic review

Krystal Iacopetta, Lyndsey E. Collins-Praino, Femke T. A. Buisman-Pijlman, Jiajun Liu, Amanda D. Hutchinson & Mark R. Hutchinson

To cite this article: Krystal Iacopetta, Lyndsey E. Collins-Praino, Femke T. A. Buisman-Pijlman, Jiajun Liu, Amanda D. Hutchinson & Mark R. Hutchinson (2018): Are the protective benefits of vitamin D in neurodegenerative disease dependent on route of administration? A systematic review, Nutritional Neuroscience, DOI: 10.1080/1028415X.2018.1493807

To link to this article: https://doi.org/10.1080/1028415X.2018.1493807

4.1 Abstract

Background: The clinical and pre-clinical exploration of the therapeutic properties of vitamin D have significantly increased in the past decade, owing to the growing associative evidence suggesting vitamin D is neuroprotective. However, whether depletion of vitamin D contributes to the onset of neurological disorders or is a symptom of neurological disease has yet to be defined. Much remains unclear about the causal role of vitamin D and the method of use and forms of vitamin D.

Objectives: We sought to quantitatively assess if neuroprotective benefits from vitamin D in neurodegenerative diseases are dependent on route of administration: comparing the effect of endogenously sourced vitamin D from UV exposure to exogenously derived vitamin D through synthetic supplementation.

Design: We systematically searched PubMed, Embase and PsycInfo databases, which included both pre-clinical and clinical studies investigating vitamin D in neurodegenerative diseases. Articles were subject to strict inclusion criteria and objectively assessed for quality. Additionally, Medline data was analysed to identify trends in topic publications and linguistic characteristics of papers.

Results: From a total of 231 screened articles, we identified 73 appropriate for review based on inclusion criteria: original studies that investigated vitamin D levels or levels of vitamin D supplementation in neurodegenerative diseases or investigated past/present sun exposure in disease cohorts. Results indicate there is insufficient evidence to comprehensively reflect on a potential neuroprotective role for vitamin D and if this was dependent on route of administration. The majority of current data supporting neuroprotective benefits from vitamin D are based on pre-clinical and observational studies. Solid evidence is lacking to support the current hypothesis that the beneficial effect

of UV exposure results from the synthesis of vitamin D. Sun exposure, independent of vitamin D production, may be protective against multiple sclerosis, Parkinson's disease and Alzheimer's disease. Yet, further research is required to elucidate the beneficial mechanism of actions of UV exposure. The literature of vitamin D and amyotrophic lateral sclerosis was limited, and no conclusions were drawn. Therefore, in cases where UV-derived vitamin D was hypothesised to be the beneficial mediator in the neuroprotective effects of sun exposure, we propose results are based only on associative evidence.

Conclusion: On the basis of this systematic review, strong recommendations regarding therapeutic benefits of Vitamin D in neurodegenerative disease cannot be made. It is unclear if vitamin D mediates a protective benefit in neurodegenerative disease or whether it is an associative marker of UV exposure, which may contribute to as of yet unidentified neuroprotective factors.

4.2 Background

Neurodegenerative disorders are incurable and debilitating conditions that result in the progressive deterioration and/or death of neurons within the brain and spinal cord (Przedborski et al., 2003). Examples include multiple sclerosis (MS), Parkinson's disease (PD), Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS) and Huntington's disease. Research suggests that vitamin D or increased sun exposure may have a positive impact in reducing the risk of development/progression of neurodegenerative disorders (Koduah et al., 2017). Relevant observational studies indicate that patients with a neurodegenerative disease tend to have lower levels of serum vitamin D compared to healthy controls. Furthermore, several studies have suggested that sun exposure attenuates disease course through influence on vitamin D production (Annweiler et al., 2013; Annweiler et al., 2011; Duan et al., 2014; Landel et al., 2016; Munger et al., 2006; Shen and Ji, 2015). Presently, the association between vitamin D and neurodegenerative disease remains to be defined and greater understanding is required to elucidate on administrative method and form of vitamin D for potential protective benefits.

Vitamin D has two distinct forms, vitamin D3 or cholecalciferol and vitamin D2 or ergocalciferol, which are derived through distinct pathways (Thacher and Clarke, 2011). The major source of vitamin D for most people is vitamin D3, generated in the skin through conversion of 7-dehydrocholesterol from skin cell membranes mediated by ultraviolet (UV) light (Bikle, 2014; Zhang and Naughton, 2010). However, changes in lifestyle and increased life expectancy have contributed to widespread vitamin D insufficiency due to a lack of adequate sun exposure, therefore increasing the use of synthetic vitamin D supplementation (Anderson, 2005; van Schoor and Lips, 2017). Vitamin D3 can also be acquired in the diet from animal sources (e.g. meat, oily fish, egg yolk and liver) via absorption through the gastrointestinal tract (Crowe et al., 2011; Zhang and Naughton,

2010). Conversely, Vitamin D2 is derived from fungi or yeast and can only be obtained exogenously through dietary intake (Japelt and Jakobsen, 2013; Zhang and Naughton, 2010). Few foods naturally have substantial quantities of vitamin D2; therefore, dietary vitamin D2 is primarily obtained through oral supplementation (Rapuri et al., 2004). Both vitamin D3 and D2 can be produced synthetically as supplements, with vitamin D2 remaining the major form of synthetic vitamin D produced commercially (Houghton and Vieth, 2006).

Presently, while vitamin D2 and D3 are typically considered equipotent at raising serum metabolite levels (Thacher and Clarke, 2011), the evidence for this is contradictory. Studies suggest that vitamin D2 is inferior to vitamin D3 in raising serum levels of 25(OH)D in all primate species, including humans (Armas et al., 2004; Marx et al., 1989; Trang et al., 1998). Moreover, the subtle differences in chemistry between the two forms of vitamin D result in lower binding affinity of vitamin D2 for the plasma vitamin D binding protein (DBP). Furthermore, an additional oxidation reaction being required for vitamin D3 to be biologically deactivated, suggesting vitamin D2 is more rapidly metabolised than vitamin D3, resulting in a faster rate of clearance from the circulation (Horst et al., 1986). However, in contrast, recent studies have presented evidence that vitamin D2 is as effective as vitamin D3 in sustaining serum vitamin D levels (Holick, 2011; Rapuri et al., 2004). Similarly, it is unclear whether vitamin D derived from oral supplementation is equivalent to vitamin D synthesised in the skin from UV exposure. In fact, we have not been able to identify a single study that addresses this question.

4.2.1 Possible mechanisms by which vitamin D may contribute protective benefit within neurodegenerative disease

Several metabolic pathways by which vitamin D may influence neurological outcomes have been proposed (see reviews (Fernandes de Abreu et al., 2009; Koduah et al., 2017)). These largely surround the hypothesis that vitamin D modulates gene transcription factors, through interactions with the vitamin D receptor (VDR), which result in neuroprotective benefits by simultaneously targeting different factors that lead to neurodegeneration (Mpandzou et al., 2016). For example, vitamin D inhibits the synthesis of inducible nitric oxide synthase (iNOS), a catalyst for the free radical nitric oxide which can damage cells (Garcion et al., 2002). Vitamin D also increases stimulation of gamma-glutamyl transpeptidase, which is an enzyme important in the synthesis of the antioxidant, glutathione, that protects from cell damage by neutralising free radicals (Garcion et al., 2002). Lastly, vitamin D can act as a neurotrophic factor, stimulating glial cells to produce nerve growth factor (NGF), glial cell derived nerve growth factor (GDNF) and neurotrophin 3 (NT3) (Neveu et al., 1994; Naveilhan et al., 1996; Musiol and Feldman, 1997), potentially beneficially contributing to growth and protection of neurons, which is critical for the prevention of diseases such as AD and PD, where neuronal cell death is prominent.

Animal models and neuronal cell culture have been instrumental in the investigation of potential molecular mechanisms by which vitamin D supplementation may confer protection against neurodegeneration, demonstrating that serum vitamin D or the active vitamin D metabolite may prevent neurotoxicity. One study found that vitamin D treated macrophage cells from AD patients had increased amyloid beta (Aβ) uptake and clearance as well as protection from apoptosis compared to untreated cells (Masoumi et al., 2009). More recently, a study using a neuroblastoma cell line also identified that vitamin D altered

Aβ production and degradation *in vitro* as well as in *ex vivo* vitamin D deficient mouse brains (Grimm et al., 2017). Effects were mediated through alterations to the Aβ–producing enzymes BACE1 and γ-secretase, suggesting that vitamin D supplementation may have therapeutic benefits for AD prevention (Grimm et al., 2017).

Another possible pathway by which vitamin D may beneficially protect against neurological disease is through modulation of the innate and adaptive immune systems (Dankers et al., 2016). Vitamin D has general immunosuppressive properties that may serve to protect the brain from inflammatory damage, such as that which occurs with MS (Prietl et al., 2013). Recent *in vivo* experimental work, using experimental autoimmune encephalomyelitis, an animal model of MS, has linked vitamin D to the metabolic and signalling pathways critical in the differentiation of naïve CD4+ T cells (Zeitelhofer et al., 2017). Vitamin D administration was linked with downregulation of pathogenic T helper (Th) cells which are critical in the development of experimental autoimmune encephalomyelitis, suggesting that vitamin D provide protective benefit in MS (Zeitelhofer et al., 2017).

Finally, animal models of PD have also demonstrated that vitamin D administration results in elevation of central dopamine levels compared to controls through enhancing GDNF expression (Smith et al., 2006; Wang et al., 2001). Animals that were pre-treated with vitamin D, prior to disease induction, showed diminished decreases in tyrosine hydroxylase (TH), the rate-limiting enzyme for the production of dopamine, within the substantia nigra, suggesting that vitamin D may have utility for the protection of dopamine neurons in PD (Sanchez et al., 2009).

4.2.2 Gaps in current evidence surrounding therapeutic benefit of vitamin D

Given that vitamin D has gained increased attention from diverse areas of biomedical research and geographical ecological evidence linking UV with disease (Grant, 2016), many groups have pursued vitamin D research and numerous studies have been published suggesting that: 1) vitamin D insufficiency leads to increased risk of neurological disease and 2) there are potential benefits of therapeutic vitamin D supplementation for these diseases (Shen and Ji, 2015). However, researchers are still elucidating whether depletion of vitamin D contributes to the onset of neurological disorders or is a symptom of neurological disease. As yet, a clear link between vitamin D and neurodegenerative disease has not been firmly established and the literature often reports conflicting results as to whether or not vitamin D has therapeutic benefits in these conditions (Annweiler et al., 2011; Kragt et al., 2009; Mosayebi et al., 2011; Suzuki et al., 2013; Zheng et al., 2018).

Additionally, two other key points of controversy in the research exist; 1) whether different forms of vitamin D are equally beneficial for neurodegenerative disease and 2) whether vitamin D derived from oral supplementation is equally beneficial as that derived directly from UV exposure.

The aim of this systematic review is firstly to establish a context for vitamin D and neurodegenerative diseases within the broad field of literature over the last few decades through preforming an analysis on MeSH terms, looking at word frequency and charting trends over time. Secondly, a traditional systematic review approach, using PRISMA guidelines, will specifically address if the neuroprotective effects of vitamin D in neurodegenerative disease are dependent on route of administration.

4.3 Methods

4.3.1 Corpus data analysis and visualisation

To identify trends in the topic of publications and linguistic characteristics of papers, Medline data was analysed. Data containing article summary data, including abstracts, titles, MeSH terms, author keywords, and journal names, were extracted from PubMed using the defined search terms as seen in Table 1 (section a. only). Trends were then analysed using the Biopython v1.69 (Cock et al., 2009) library via Python 2.7.

Abstracts were word tokenized (split into single words) and converted to lowercase. Stop words (common grammar terms like “and” “is” “was”) and punctuation were removed. All words were converted to word-stems (i.e. running -> run) using the LancasterStemmer algorithm in Natural Language Toolkit (NKLT).

The bag-of-words approach was then used to sum incidences of each word in the vocabulary for each document, yielding a sparse matrix with word counts per word, per abstract. The bag-of-words transforms the dataset, where each row represents a separate document (abstract), and each column contains words mentioned across all documents. Each cell in the dataset records the frequency of each word in the document, thus generating a sparse matrix.

Term frequency inverse document frequency (TFIDF) transformation was further applied to correct total term frequency by accounting for the number of documents that mention each term. From this analysis, higher weights were given to terms that were repeatedly mentioned across documents rather than the number of times within documents. This was implemented using the Transformer function in scikit.learn.

Nouns were extracted from the sentence structure using part of speech tagging via pos_tag in NLTK. Noun tagged words were identified as singular nouns (nouns should all

be singular after word stemming). Word frequency was performed on / keywords/ MeSH terms/ authors/ nouns from stemmed bag-of-words transformed abstracts. Noun trends were compiled from the most frequent nouns (see graph) identified from abstracts from 1964-2015.

To investigate the contextual use of descriptors of direction-weighted words, such as “increase,” “decrease” and “association”, the body of abstracts was first searched for 15-word phrases containing the target words using concordance analysis, which, as much as possible (with the exception of end of text or abstract), tries to place the target word in the middle of the 15-word phrase. For example, "an effective treatment strategy to decrease fatigue and improve quality of life in patients with MS. Objective” (Achiron et al., 2015).

N-gram analysis, which takes a body of text and breaks it up into “n” word chunks, was then applied to turn these 15-word phrases into bi-grams and tri-grams. Bi-grams and tri-grams were thus obtained from each phrase and passed through a frequency analysis.

Word2Vec approximates word similarity through contextual information via eigen distances between each word. For example, similarities between “king” and “male” would hold true for “queen” and “female” due to the relative context (distance) between these words across documents. Word2vec was applied to stemmed word vectors using the Word2Vec function from gensim. Most similar words to each target word were obtained using the most_similar function in gensim.

4.3.2 Search strategy for systematic review

The following systematic review was prepared following the PRISMA approach to ensure quality of research and minimise the risk of bias (McInnes et al., 2018). In order to identify all eligible studies, we performed a literature search of PubMed, Embase and PsychINFO databases for all papers published in English up until April 2016. Search terms

included “vitamin D” and related words combined with those used to identify neurodegenerative disease in the titles or abstracts. No limits were used in the searches. The exact search strings used are depicted in Table 1. In addition, the reference list of each included article was manually searched to identify additional eligible studies.

4.3.3 Systematic review publication selection criteria

The search results were imported into EndNote and duplicates were deleted. The potentially eligible studies were identified by screening titles and/or abstracts, and/or the full text of references. Studies were eligible for inclusion if they (i) were original studies investigating an association between vitamin D levels or vitamin D supplementation with neurodegenerative disease and reported a neurological outcome (for example, improved disease symptoms); or (ii) looked at past or present sun exposure in neurodegenerative disease cohorts. Studies were excluded if they (i) were about vitamin D but did not have a neurological/clinical outcome; (ii) did not adequately provide a measure of vitamin D or specifically state month/time spent outdoors; (iii) pilot studies that did not have significant power to measure clinical outcomes; (iv) were case reports/expert options; or (v) were review articles.

4.3.4 Systematic review publication data extraction

The following data were extracted from each included study: (i) study characteristics (first author, year of publication, study design); (ii) principal aim and hypothesis and (iii) whether the outcome supported the hypothesis. Vitamin D was assessed for a protective effect.

Table 1. Search terms

a. PubMed	
	Vitamin d* [tw] OR vitamin D [mh] OR vit D*[tw] OR ergocalciferol [tw] OR cholecalciferol [tw]
OR	Ultraviolet [tw] OR ultra violet [tw] OR UV [tw] OR UV-A [tw] OR UV-B [tw] OR sunlight [tw] OR sun exposure [tw]
AND	Parkinsonian disorders [mh] OR parkinson* [tw] OR alzheimer* [tw] OR dementia [tw] OR dementia [mh] OR lewy body dementia [tw] OR huntington* [tw] OR motor neuron disease [mh] OR motor neuron disease [tw] OR multiple sclerosis [mh] OR multiple sclerosis [tw] OR prion disease [mh] OR prion disease [tw] OR progressive supranuclear palsy [tw] OR PSP [tw] OR vascular dementia [mh] OR vascular dementia [tw] OR multiple system atrophy [mh] OR multiple system atrophy [tw] OR spinocerebellar ataxia [mh] OR spinocerebellar ataxia [tw] OR spinal muscular atrophy [tw] OR friedreich* [tw]
b. Embase	
	‘Vitamin d’:ab,ti OR ‘vitamin D’:ab,ti OR ‘Vit D’:ab,ti OR 'ergocalciferol':ab,ti OR cholecalciferol:ab,ti
OR	'ultraviolet radiation’:ab,ti OR 'ultraviolet radiation':ab,ti OR ultra violet:ab,ti OR UV OR UV-A OR UV-B OR 'sunlight':ab,ti OR 'sunlight':ab,ti OR 'sun exposure':ab,ti
AND	‘Parkinsonian disorders’ OR parkinson*:ab,ti OR ‘dementia’:ab,ti OR ‘diffuse Lewy body disease':ab,ti OR Huntington*:ab,ti OR 'motor neuron disease':ab,ti OR ‘multiple sclerosis’:ab,ti OR 'prion disease’:ab,ti OR 'progressive supranuclear palsy':ab,ti OR 'multiinfarct dementia':ab,ti OR 'Shy drager syndrome':ab,ti OR 'spinocerebellar degeneration':ab,ti OR 'spinal muscular atrophy’:ab,ti OR 'friedreich ataxia':ab,ti
c. PsychINFO	
	Vitamin d OR vitamin D OR vit D OR ergocalciferol OR cholecalciferol
OR	Ultraviolet radiation OR ultraviolet radiation OR Ultra violet OR UV OR UV-A OR UV-B OR sunlight OR sunlight OR sun exposure
AND	Parkinsonian disorders OR Parkinson* OR dementia OR diffuse Lewy body disease OR Huntington* OR motor neuron disease OR multiple sclerosis OR prion disease OR 'progressive supranuclear palsy OR multiinfarct dementia OR shy drager syndrome OR spinocerebellar degeneration OR spinal muscular atrophy OR Friedreich ataxia

4.4 Results

4.4.1 Corpus data analysis summary

4.4.1.1 Description of the vitamin D field of research

To establish the context in which this systematic review was conducted, an analysis of MeSH term frequency was performed to provide an overview of the state of play and trends in the literature (Figure 4.1a). In the last three decades, there have been more human studies compared to pre-clinical studies, with females featured in marginally more papers than males. To date, there has been a predominance of UV rather than vitamin D and dose response studies, with risk factors, disease progression, retrospective and cohort studies having higher prevalence than treatment outcomes from PubMed's classification system. These data perhaps indicate higher associative rather than experimental or clinical studies.

Deeper natural language processing analysis from the abstract corpus allowed exploration of the frequency of the use of nouns in abstracts yielding high rates of the use of "vitamin" and "d" and terms most frequent throughout most abstracts. This is substantiated by the "bag of words" (BOW) approach to vectorised language processing and term TFIDF analysis. To correct for potential small groups of abstracts that had higher incidences of particular words, a TFIDF transformation was used on the BOW matrix. This yielded a reduction of gaps between "vitamin" and "MS", the second most mentioned term (Figure 4.1c), thus implying that MS is an important descriptor for a majority of abstracts, and hence the predominance of the field focusing on this pathology.

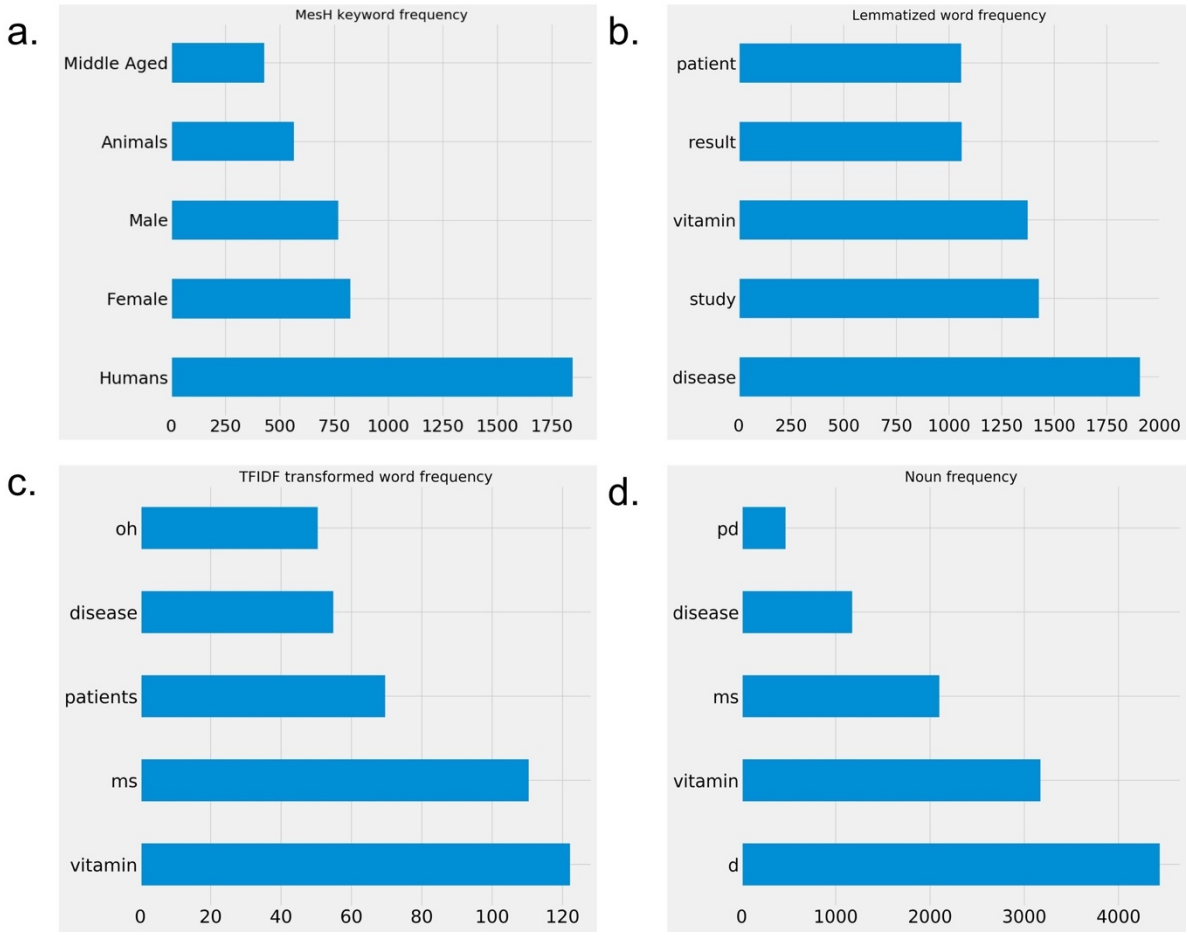


Figure 4.1. Frequency distributions of top 5 MeSH keywords (a.) Lemmatised words (b.) Term frequency inverse document frequency transformed words (c.) and noun frequency (d.).

Finally, to visualise the vitamin D research trends over time, we plot incidence of selected nouns across time (Figure 4.2). “p”(value) was plotted as a baseline mention, as it should be a stable descriptor of papers, i.e. a proportional number of papers would mention p-values. Frequency distribution of nouns increased in general across time, with the exception of “dopamine,”which had lower values in recent years. Mentions of “(vitamin) d” had the highest growth across nouns investigated, followed by MS. There were also increased trends in “disease”, “PD” (Parkinson's disease), “AD” (Alzheimer's disease) and “(vitamin) d3”, showing potential increasing correlations between vitamin d research and

disease states. Of note is the profound increase of publications in the field in the last decade, highlighting the timely nature of this systematic review.

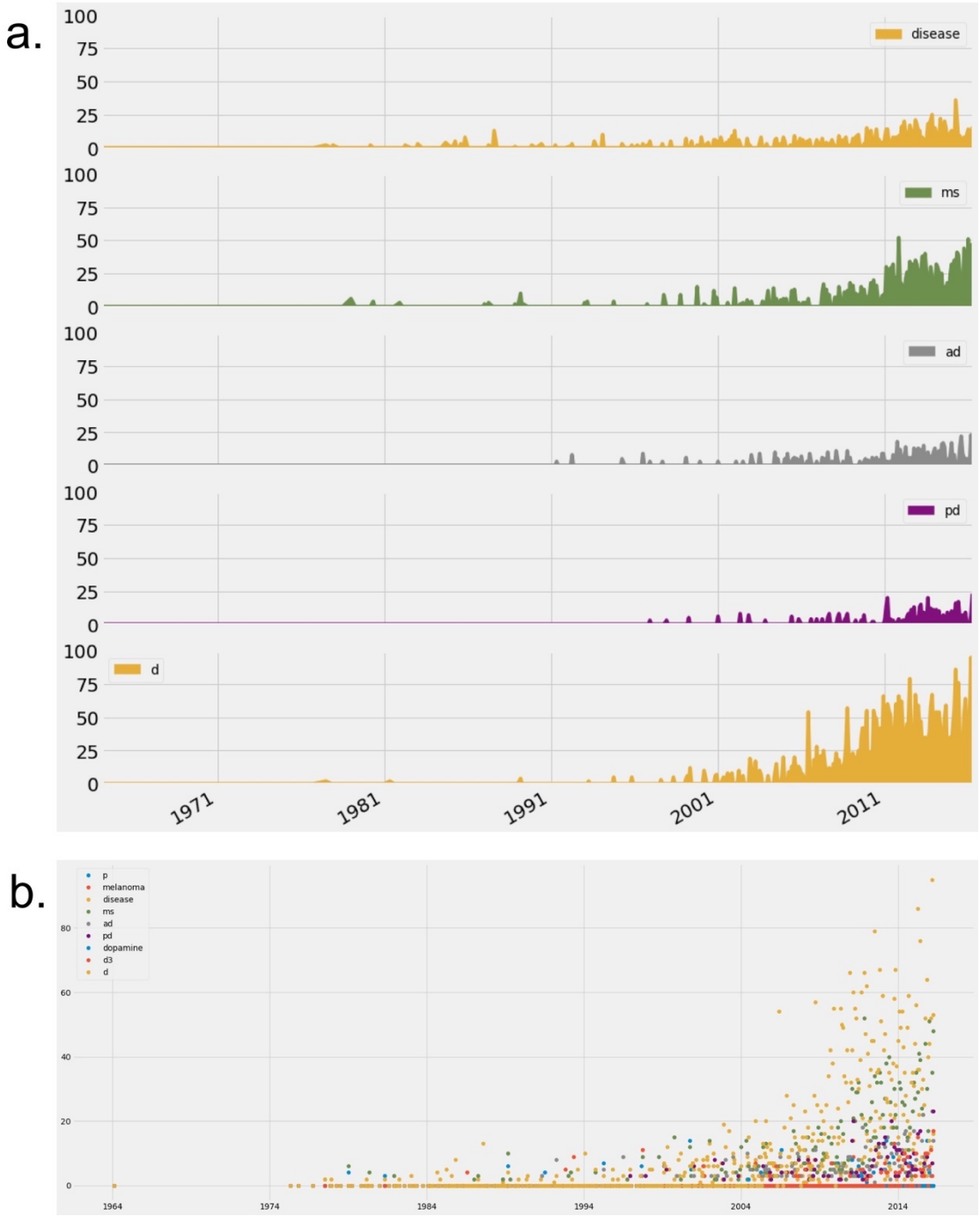


Figure 4.2. Trends in selected noun mentions in abstracts across time.

4.4.2 Systematic review search outcomes

The outcomes of the PRISMA screening process are provided in Figure 4.3. The initial search yielded 5107 articles. Of these, 2440 were immediately excluded (1660 were identified as duplicates and 780 were review papers), leaving 2667 articles for screening based on title and abstract. An additional 2436 were excluded, as they were not relevant to the research question. 231 articles underwent full text review, resulting in 73 articles eligible for inclusion. Of these, 12 articles were flagged as having a potential conflict of interest, which have been outlined in each table.

To aid in the targeted review and interpretation of the broad fields covered by this systematic review, eligible articles were then segregated into the following disease types for further analysis and discussion; MS (n=46); PD (n=12); AD (n=10) and ALS (n=5). All articles were separated into clinical and pre-clinical subgroups for further analysis. Study characteristics are summarised in Tables 2-10.

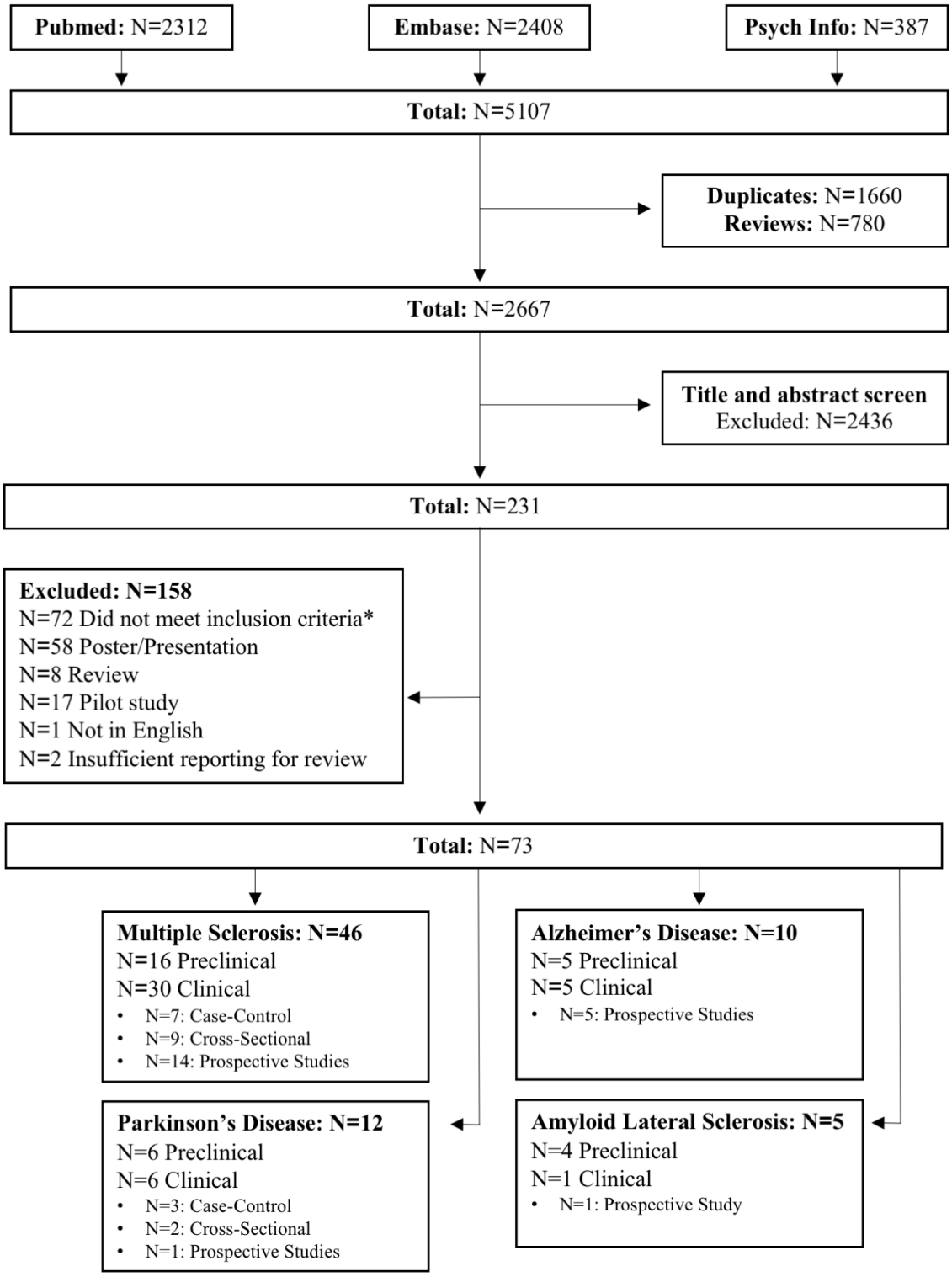


Figure 4.3. Flow diagram outlining the study selection process. *Inclusion criteria were original studies that investigated vitamin D levels or vitamin D supplementation in neurodegenerative diseases or investigated past/present sun exposure in disease cohorts.

4.4.3 Multiple sclerosis

A total of 46 articles relevant to MS were included from the search. Of these, 21 articles reported a protective benefit improving disease outcomes that was attributed to vitamin D. The study characteristics from the identified articles relevant to MS can be found in Tables 2-5. Tables were organised according to study type; Table 2 lists pre-clinical studies (n=16); Table 3 documents retrospective case control studies (n=7); Table 4 details cross-sectional studies (n=9) and Table 5 lists the prospective clinical trials (n=14).

4.4.3.1 Pre-clinical studies

The 16 pre-clinical studies identified herein, predominately induced MS symptoms in rodents using the experimental autoimmune encephalomyelitis (EAE) model, injecting antigenic compounds that cause demyelination of neurons creating an MS-like pathology (Adzemovic et al., 2013; Becklund et al., 2010; Farias et al., 2013; Garcion et al., 2003; Nashold et al., 2000; Nashold et al., 2013; Nataf et al., 1996; Pedersen et al., 2007; Sloka et al., 2015; Soleimani et al., 2014; Spach and Hayes, 2005; Spach et al., 2006; Wang et al., 2015b; Wang et al., 2012; Wang et al., 2013). One of the pre-clinical studies used the toxin induced demyelination model, injecting lysophosphatidyl choline (LPC) in the hippocampus (Tarbali and Khezri, 2016). A potential relationship between MS and vitamin D was then investigated by either oral/intraperitoneal or gavage vitamin D3 metabolite supplementation (n=11), (Adzemovic et al., 2013; Farias et al., 2013; Garcion et al., 2003; Nashold et al., 2000; Nashold et al., 2013; Nataf et al., 1996; Pedersen et al., 2007; Sloka et al., 2015; Soleimani et al., 2014; Spach and Hayes, 2005; Tarbali and Khezri, 2016) through UVR induced epidermal vitamin D production (n=3), (Becklund et al., 2010; Wang et al., 2015b; Wang et al., 2013) and lastly using knockout animals devoid of either vitamin D receptor or enzymes that convert active vitamin D3 (n=1) (Wang et al., 2012).

Of the eleven studies using vitamin D supplementation, three studies specifically looked at the protective effects of vitamin D prior to EAE induction, manipulating vitamin D3 levels in the diet before EAE inoculation (Adzemovic et al., 2013; Spach and Hayes, 2005; Spach et al., 2006). In the first two studies, animals were treated with either 0, 2, 10IU/gram (Adzemovic et al., 2013) or 0, 1, 5µg/day (Spach and Hayes, 2005) of vitamin D3 for 3-8 and 4 weeks respectively. These two studies identified that vitamin D therapy was beneficial in preventing or reducing clinical symptoms of MS in the EAE model only in the juvenile/adolescent age groups (Adzemovic et al., 2013) and in female mice (Spach and Hayes, 2005). Other tested groups that were not significant include adult animals that were supplemented with vitamin D3 for 8 weeks (Adzemovic et al., 2013) and ovariectomised or intact or castrated males (Spach and Hayes, 2005). In a subsequent study by Spach et al (2006), using a similar protocol of 1 ng/day vitamin D3 for 4 weeks prior to EAE induction, similar results were observed with a reduced effect only in the female mice. However, when given pre-treatment with active vitamin D (50 ng for females and 100 ng for males) before EAE induction, both males and females had lower incidence of EAE and improved clinical outcomes (Spach et al., 2006). The remaining nine studies provided vitamin D treatment at varying stages of disease onset as detailed in table 4 (Farias et al., 2013; Garcion et al., 2003; Nashold et al., 2000; Nashold et al., 2013; Nataf et al., 1996; Pedersen et al., 2007; Sloka et al., 2015; Soleimani et al., 2014; Spach et al., 2006; Tarbali and Khezri, 2016). Although outcome measures were different, including clinical score analysis, measure of inflammatory markers and the degree of re-myelination, all nine studies reported that vitamin D therapy improved disease outcomes or reduced inflammation in EAE models of MS when compared to control animals. It is noteworthy, that one study, which supplemented vitamin D via both gavage and intraperitoneal routes, found vitamin D to be protective only when administered at higher doses and via gavage.

Low dose gavage and intraperitoneal injections had no significant benefit on clinical outcomes (Farias et al., 2013).

Three pre-clinical studies investigated the potential protective effects from increasing vitamin D via UVR in suppressing disease severity in the EAE mouse model of MS. All three studies irradiated mice with either 2.5, 5 or 10kj/m^2 of UVR for 7 days prior to EAE immunisation and then continued UVR exposure for up to 30 days (Becklund et al., 2010; Wang et al., 2015b; Wang et al., 2013). Although UV-B suppressed EAE, all three studies concluded that the observed UV-B mediated suppression occurred independently of vitamin D, as circulating serum vitamin D levels were not elevated. Therefore, vitamin D3 was determined as not being protective in these studies, contrary to the conclusions drawn from the previously reviewed pharmacological manipulation studies.

Interestingly, in the study using genetic knockout animals, vitamin D had the opposite effect and was reported as necessary for the development of EAE (Wang et al., 2012). Genetically modified mice lacking the vitamin D receptor showed markedly suppressed EAE, and mice with insufficient vitamin D had partially suppressed EAE when compared with wild type animals. These unexpected results indicate that vitamin D may have a potential role in the development of elements of the immune system that are required for the autoimmune disease EAE.

4.4.3.2 Case-control studies

This review identified seven case-control studies eligible for inclusion in the analysis and these are summarised in Table 2b. The case-control studies retrospectively investigated lifestyle factors such as sun exposure (Baarnhielm et al., 2012; Bjornevik et al., 2014; Dalmay et al., 2010; Espinosa-Ramirez et al., 2014; Islam et al., 2007; Kampman

et al., 2007; Van Der Mei et al., 2003) and diet (Kampman et al., 2007) in MS patients (n=2371) compared to controls (n=4445) and investigated whether activities associated with increased vitamin D were protective in decreasing the risk of MS. All studies questioned participants on specific time periods during childhood and adolescence. However, one study also reported on sun exposure in the last 5 years (Espinosa-Ramirez et al., 2014). Questionnaires were completed comprised of self-report measures (Kampman et al., 2007; Bjornevik et al., 2014) or through interviews (Dalmay et al., 2010; Espinosa-Ramirez et al., 2014; Islam et al., 2007; Van Der Mei et al., 2003).

The majority (n=6) of these case-control articles reported that increased sun exposure and time outdoors during childhood and adolescence was associated with decreased risk of developing MS (Bjornevik et al., 2014; Dalmay et al., 2010; Islam et al., 2007; Kampman et al., 2007; Van Der Mei et al., 2003). Two studies demonstrated a dose-response like effect, in which higher sun exposure was associated with decreased relative risk (Islam et al., 2007; Kampman et al., 2007). These data suggest that sun exposure, postulated to be mediated by elevating vitamin D levels, was beneficial in reducing the risk of disease. Contrary to the results reported in the three UVR pre-clinical studies that suggest UVR had a protective effect independent from vitamin D. Another study, investigating UV exposure in the past 5 years found similar results, reporting increased UVR decreased the risk of MS (Baarnhielm et al., 2012). This study also suggested that the protective benefit from UV may be mediated by pathways independent of vitamin D as adjusting for a variety of confounding factors, including vitamin D serum levels at inclusion of the study, did not affect the strength of the association (Baarnhielm et al., 2012). Two studies demonstrated a dose-response like effect, in which higher sun exposure was associated with decreased relative risk (Islam et al., 2007; Kampman et al., 2007). The remaining case-control study found no association between past or current sun exposure and the risk of development of

MS and reported that MS patients spent more time in sunlight compared to controls both during adolescence and in the past 5 years.

4.4.3.3 Cross-sectional studies

The search identified nine cross-sectional studies eligible for inclusion that were relevant to MS (see table 4). Three cross-sectional studies investigated MS cohorts by comparing self-report data of life style factors, including past and present sun exposure, with geographic location/latitude (McDowell et al., 2011a; McDowell et al., 2011b) or residential solar estimates (Jelinek et al., 2015). All studies showed a positive correlation between increased past sun exposure and improved disease outcomes, concluding that sun exposure was likely to be a key environmental factor to modulate disease. One cross-sectional study compared relapsing MS with progressive MS, reporting vitamin D related sun exposure influenced disease types independently. Increased sun exposure in the past 10 years was protective in relapsing MS, whereas increased sun sensitivity or the likeliness to burn was associated with increased hazard in progressive MS (D'Hooghe M et al., 2012).

The five remaining studies similarly used questionnaire information on lifestyle factors related to vitamin D exposure in MS cohorts, but also measured current serum vitamin D metabolites. Two studies found support for a protective effect of vitamin D (Van Der Mei et al., 2007; Mandia et al., 2014), while one study reported mixed results (Lucas et al., 2011) and two studies found no association between vitamin D and disease (Hejazi et al., 2014; Gelfand et al., 2011).

Of the two studies that showed a protective benefit of vitamin D, one study specifically looked at the effect of vitamin D on disease severity rather than risk and found that increased sun exposure and adequate vitamin D was associated with relatively mild MS for a sample population of 131 MS patients (Mandia et al., 2014). Consistent with this,

the second article in support of vitamin D showed that lower levels of serum vitamin D were associated with reduced sun exposure and increased disability in MS patients compared to control participants (Van Der Mei et al., 2007). The article which reported mixed results reported that increased lifetime sun exposure was associated with reduced risk of MS. However, vitamin D and current sun exposure were independent of each other, suggesting that another possible UV-related factor was involved in protection from MS disease aetiology (Lucas et al., 2011) consistent with the three pre-clinical studies that used UVR (Wang et al., 2015b; Wang et al., 2012; Wang et al., 2013). Of the two cross-sectional studies that found no association between vitamin D and disease, the first looked at self-report data of dietary intake of multiple vitamins, including vitamin D, and sun exposure but found no significant difference between patients and controls. Serum sampling from all participants showed low antioxidant level and vitamin D insufficiency (Hejazi et al., 2014). The final cross-sectional study investigated the correlation between disease severity and circulating vitamin D within an African American study population. The study reported that vitamin D was not associated with disease severity, as MS patients had higher serum vitamin D compared to control subjects, but this was primarily attributed to differences in place of dwelling associated with climate and geography (Gelfand et al., 2011).

4.4.3.4 Prospective studies

Fourteen prospective clinical articles were identified as eligible for inclusion; summary data can be observed in table 5. Five (n=5) longitudinal cohort studies (Kragt et al., 2009; Knippenberg et al., 2014; Loken-Amsrud et al., 2012; Munger et al., 2004; Simpson Jr et al., 2010), eight (n=8) double blind randomised controlled trials (Achiron et al., 2015; Derakhshandi et al., 2013; Golan et al., 2013; Kampman et al., 2012; Mosayebi et al., 2011; Shaygannejad et al., 2012; Stein et al., 2011a) and one (n=1) non-blinded clinical trial (Farsani et al., 2015) were included.

Three of the five longitudinal cohort studies measured serum vitamin D levels of MS patients every 6 months, with follow up periods of 6 months, (Kragt et al., 2009) 2.3 years (Knippenberg et al., 2014) and 3 years (Simpson Jr et al., 2010). The first study (Kragt et al., 2009) compared MS patients with controls, whereas the latter two (Knippenberg et al., 2014; Simpson Jr et al., 2010) only included MS patients. The first two studies reported that higher circulating levels of vitamin D were associated with decreased MS-related disability and relapse rate (Kragt et al., 2009; Simpson Jr et al., 2010). The third longitudinal study examined separate disease characteristics including depression, anxiety, fatigue and cognition and found that higher reported sun exposure, rather than vitamin D levels, was associated with improved depressive and fatigue symptoms. No effect was observed between sun exposure, vitamin D and cognition (Knippenberg et al., 2014).

The remaining two longitudinal cohort studies reported mixed results (Loken-Amsrud et al., 2012; Munger et al., 2004). One study investigated vitamin D status by correlating levels to lesion development, determined by MRI, for 6 months preceding interferon-β (IFN-β) treatment and then an additional 18 months thereafter. Vitamin D was associated with a reduced odds ratio for lesion development prior to IFN-β, however, once treatment began there was no association between disease activity and vitamin D (Loken-Amsrud et al., 2012). The final study used data acquired from the nurses' health study cohorts, in which food frequency/dietary intake questionnaires were distributed every four years. During the 20-year follow up period 173 cases of MS had developed. It was identified that women who supplemented with vitamin D had a 40% lower risk of developing MS compared to women who did not take supplementation. No protective benefit was observed from dietary vitamin D intake and the risk of developing MS (Munger et al., 2004).

The non-blinded clinical trial reported that intramuscular supplementation with vitamin D in MS patients resulted in a reduced mean average of expanded disability status scale (EDSS), attributed to increased interleukin-10 gene expression. This study was performed over an 8-week period and serum samples were analysed for gene expression and markers of inflammation against serum samples from health controls. This data was later correlated with EDSS scores (Farsani et al., 2015).

The remaining eight randomised controlled trials were all double-blinded and predominately used supplementation with oral vitamin D3 capsules (Achiron et al., 2015; Derakhshandi et al., 2013; Golan et al., 2013; Kampman et al., 2012; Shaygannejad et al., 2012; Soilu-Hänninen et al., 2012). However, one study supplemented via intramuscular injection (Mosayebi et al., 2011) and one study supplemented with vitamin D2 (Stein et al., 2011a). All except one study, that reported an improvement in fatigue impact score (Achiron et al., 2015), found no therapeutic advantage of vitamin D therapy on clinical outcomes of MS compared to controls. Five studies looked specifically at the EDSS and found no effect between treatment groups; (Achiron et al., 2015; Golan et al., 2013; Mosayebi et al., 2011; Shaygannejad et al., 2012; Soilu-Hänninen et al., 2012); other measures included relapse rate (Golan et al., 2013; Shaygannejad et al., 2012) and MRI lesion size (Soilu-Hänninen et al., 2012; Stein et al., 2011a). All studies had relatively low sample sizes, ranging from 11-80 participants. Importantly, results were not suggestive of a vitamin D related protective effect on MS outcomes.

In summary, these data indicate that a clear role for a neuroprotective benefit from vitamin D in MS cannot be established. The pre-clinical and observational data are in support for protective/therapeutic benefit from vitamin D, however, this same positive trend is not observed within results from prospective clinical trials of MS patients. Furthermore, there is only minimal data supporting the UV-vitamin D conversion link for

beneficial actions of sun exposure in MS. Rather, there appears to be a sun or UV specific factor–independent of vitamin D that may mediate the sun exposure benefits related to MS.

4.4.4 Parkinson's disease

Tables 6 and 7**,** respectively, describe the pre-clinical and clinical study characteristics of all identified studies from the described search specific to PD. Twelve studies were included, with nine articles in support of a positive or beneficial effect of vitamin D in either decreasing disease risk or improving clinical outcomes in PD. The twelve articles were categorised as either pre-clinical studies using animal models of disease (n=6) or clinical studies involving retrospective case control studies (n=3), cross-sectional studies (n=2) and prospective controlled trials (n=1).

4.4.4.1 Pre-clinical studies

The six pre-clinical studies applied a vitamin D intervention to rodent models of PD, in which dopaminergic neurons in the substantia nigra were lesioned with neurotoxin, either 6-hydroxydopamine (6-OHDA) or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyidine (MPTP), inducing PD-like symptoms, in order to investigate the effects of vitamin D on dopamine (DA) neuron integrity as indicated by TH expression. With the exception of one study, that dosed with 100µg 6-OHDA (Smith et al., 2006), all lesions were induced with similar doses. Studies using 6-OHDA (Cass et al., 2014; Kim et al., 2006; Sanchez et al., 2009; Wang et al., 2001) used either 8ug or 12ug compared to studies using MPTP (Dean et al., 2012; Kim et al., 2006) that dosed with 15 or 20g. Four studies supplemented vitamin D3 (1µg/ml/kg/day) through intraperitoneal injections for seven days prior to the neurotoxic lesion (Kim et al., 2006; Sanchez et al., 2009; Smith et al., 2006; Wang et al., 2001). The study by Smith et al. (2009) also included a long-term treatment group that received a further 28 days of vitamin D treatment post lesion. One study supplemented

vitamin D3 (either 1 or 0.3μg/ml/kg/day) via subcutaneous injection for eight days 4 weeks post lesion (Cass et al., 2014). The final study restricted vitamin D intake with a vitamin D depleted diet for 6 weeks before exposing animals to the neurotoxin MPTP (Dean et al., 2012).

All five studies that supplemented with vitamin D3 reported increased DA or TH levels within the substantia nigra tissue, indicting reduced neuronal damage sustained from neurotoxic injury; functional tests were not performed (Cass et al., 2014; Kim et al., 2006; Sanchez et al., 2009; Smith et al., 2006; Wang et al., 2001). Vitamin D was determined as protective against the onset/severity of PD in rodent models (Cass et al., 2014; Kim et al., 2006; Sanchez et al., 2009; Smith et al., 2006; Wang et al., 2001). However, of these, one study did report mixed results, finding vitamin D treatment was only protective in the long-term group that received vitamin D supplementation both before and after lesion (Smith et al., 2006). This study also used different neurotoxins and species between each group, 6-OHDA in rats for the short-term group and MPTP in mice for the long-term group, which makes it difficult to draw conclusions between treatment groups. Importantly, however, the study that eliminated vitamin D from the diet prior to MPTP injury observed no difference in the susceptibility of DA neurons to injury and the development of PD symptoms between groups (Dean et al., 2012) indicating that vitamin D deficiency may not contribute to the development of disease.

4.4.4.2 Case-control studies

The three case control studies retrospectively analysed lifestyle factors and dietary habits as indictors of vitamin D levels in PD patients, with a total population size of 949 patients and 1426 control participants. Of these, only one study identified that increased vitamin D levels may be protective by decreasing the risk of developing PD. This study reported that increased outdoor physical activity and higher total vitamin D intake were

inversely associated with the risk of PD by calculating the odds ratio for separate quartiles (of hours/week and intake/day), showing significant decreasing trends of P=0.002 and P=0.011 respectively (Zhu et al., 2014). However, when investigating a potential joint effect of vitamin D and physical activity on risk of PD, significance was lost, suggesting that both outdoor physical activity (and, hence, UV exposure) and vitamin D may act independently (Zhu et al., 2014). The remaining two studies did not report significant correlations between indicators of vitamin D levels and the risk of PD between patient and control subjects, using occupational sunlight as an indicator of vitamin D in one study (Kwon et al., 2013) and the consumption of dairy products containing vitamin D in the other (Miyake et al., 2011).

4.4.4.3 Cross-sectional studies

The two cross-sectional studies had a pooled population of 1099 patients and 730 control participants. These two studies primarily measured vitamin D status of participants by sampling serum metabolites of vitamin D2 and D3 (Wang et al., 2016) or D3 only (Wang et al., 2015a; Wang et al., 2016) to investigate the association between serum vitamin D and PD within the sample population. One study also recorded sunlight exposure and vitamin D intake from participants (Wang et al., 2015a). Both studies reported an association between vitamin D deficiency and PD, as PD patients had lower serum vitamin D, irrespective of D2 or D3, than controls, indicating that low serum vitamin D levels are significantly associated with an increased presence of PD in cross-sectional studies.

4.4.4.4 Prospective study

The most robust study, the prospective clinical intervention study, supported a neuroprotective effect of vitamin D in existing PD patients. The effect of vitamin D3 supplementation on the disease progression of PD was investigated using a 12-month

double blind, placebo-controlled trial in 104 PD patients (Suzuki et al., 2013). Patients were divided into a treatment group (n=56) receiving 1200 IU of vitamin D3 daily in the form of an oral capsule and a control (n=58) receiving a matched placebo. The average time since diagnosis for each group was 24 months and 13 months, respectively. Importantly, it was found that the vitamin D3 supplementation group had significantly reduced symptom scores, determined by treating neurologists, on both the modified Hoehn and Yahr (H&Y) scale and the Unified Parkinson's disease rating scale (UPDRS), which are used to determine severity of symptoms in PD patients.

In summary, the data suggest a stronger association between adequate vitamin D and reduced risk of PD compared with the MS results; however, few studies met the inclusion criteria. Moreover, pre-clinical study designs varied significantly in PD models as well as treatment regimens making it difficult to draw hard conclusions. Retrospective study designs only show that vitamin D deficiency is associated with disease, but cannot provide evidence directly connecting vitamin D with disease risk or progression. Similarly, although the pre-clinical data showed that vitamin D therapy may improve disease outcomes, the lack of ample clinical data (there has only been the single prospective clinical intervention trial to date) means it is not possible yet to determine if this can be successfully translated clinically. Additionally, results from this systematic review cannot determine whether the source of vitamin D (i.e. oral supplementation or UV exposure) is influential in disease onset/progression.

4.4.5 Alzheimer's disease and dementia

Tables 8 and 9 detail the study characteristics of all pre-clinical and clinical studies identified from the search specific to AD. A total of ten studies were included in the final analysis. Of these, eight studies supported vitamin D having a protective effect in the

progression of AD and the remaining two studies did not conclude a positive benefit from vitamin D treatment. Articles were separated into pre-clinical studies (n=5) and clinical studies (n=5), including three cohort studies and two randomised controlled trials.

4.4.5.1 Pre-clinical studies

The five pre-clinical studies used rodent models of AD; two studies used transgenic animals with characteristic gene defects consistent with familial AD; one study used the AβPP-PS1 transgenic mouse model (Yu et al., 2011) whereas the remaining two used intracerebro-ventricular (ICV) injection of Aβ 1-42 to induce AD-like symptoms (Taghizadeh et al., 2014; Taghizadeh et al., 2011). All five pre-clinical studies looked at the effects of vitamin D on AD by adjusting levels of vitamin D3 available in the diet; using normal rodent chow with standard vitamin D levels, or either rodent chow fortified with increased vitamin D or chow that had been depleted of vitamin D.

The three studies using transgenic rodent models of AD all investigated long-term vitamin D treatment outcomes on cognitive and memory performance, brain inflammation and amyloid plaque formation in transgenic animals compared to wild-type control animals. All studies reported positive outcomes from vitamin D treatment at conclusion of their studies (Bennett et al., 2013; Landel et al., 2016; Yu et al., 2011). The first study, over a 7-month period, fed chow enriched with vitamin D2-button mushroom (VDM) or depleted chow to APPswe/PS1dE9 mice, and demonstrated that VDM contributed to significantly improved scores in memory and learning tests in wild-type animals and to a lesser extent VDM transgenic animals (Bennett et al., 2013). There were also decreases in the number of plaques and markers of inflammation in transgenic mice fed the VDM diet compared to the control diet (Bennett et al., 2013). Similarly, the study by Landel et al. 2016, in which 5XFAD transgenic mice were fed either high or low vitamin D diets for a period of 8 months, also reported that vitamin D improved functional outcomes compared

to control, with the wild type animals showing the highest improvement in cognitive tasks as well as decreased signs of inflammation (Landel et al., 2016). Finally, the third study assessed the impact of vitamin D on the formation of amyloid plaques in the AβPP-PS1 transgenic mice and showed that increased vitamin D3 levels (12IU/g) correlated with reduced amyloid plaque formation in this model (Yu et al., 2011).

The final two pre-clinical studies, performed by the same research group, used intracerebroventricular injection of Aβ 1-42 to induce AD like symptoms. One study reported vitamin D3 to be beneficial by demonstrating that oral vitamin D3 supplementation (200ng/rat/d) restored suppressed synaptic plasticity in Aβ 1-42 -induced rats (Taghizadeh et al., 2014). Conversely, an earlier study using higher doses of vitamin D3 (1000ng VD3/100g chow) to ameliorate deficits induced by Aβ 1-42 in spatial performance found no significant benefit from vitamin D treatment (Taghizadeh et al., 2011).

4.4.5.2 Longitudinal cohort studies

The search identified three longitudinal cohort studies investigating vitamin D and AD that were eligible for inclusion. Two studies concluded that low serum vitamin D levels were positively correlated with the risk of developing AD and Non-Alzheimer's dementia (NAD) (Annweiler et al., 2011; Afzal et al., 2014). The third study reported dietary vitamin D intake at baseline was inversely associated with the onset of AD, suggesting that lower levels of vitamin D intake at baseline was associated with lower risk of AD (Annweiler et al., 2012).

The first study analysed stored baseline plasma samples, taken from participants (n=10,118) from the Copenhagen City heart study, for levels of the vitamin D metabolite 25(OH)D in order to investigate the association between serum vitamin D and the later

development of either AD or vascular dementia (Afzal et al., 2014). During the 30-year follow up period, 418 people were diagnosed with AD and 92 people with vascular dementia. The combined endpoint of AD and vascular dementia showed that reduced plasma 25(OH)D at baseline was associated with increased risk of disease within the cohort population, suggesting that vitamin D is protective and reduces the risk of disease. The second cohort study, consisting of only female participants, measured serum vitamin D levels to determine if vitamin D deficiency was predicative of developing NAD within a 7-year time frame (Annweiler et al., 2011). This study analysed stored serum samples from 40 participants in the EPIDOS Toulouse longitudinal study. Participants were divided into two groups, determined by level of vitamin D deficiency. At the end of the 7-year period, data on cognitive status (i.e. no dementia, or AD, or NAD) were reported and correlated to baseline vitamin D metabolite levels. This study demonstrated that baseline serum vitamin D deficiency was predicative of the onset of NAD within a 7-year period among older women, also supporting that vitamin D may have a protective effect in preventing disease. The final study was published by the same research group again using the EPIDOS data set, however, this study investigated if baseline dietary vitamin D intake was an independent predictor of the onset of dementia among the cohort of women (Annweiler et al., 2012). This study categorised the participants into three groups according to the onset of dementia (no dementia, Alzheimer's Disease or other dementias) within the 7-year time frame. Baseline dietary vitamin D was then estimated for each participant from the self-report food frequency questionnaire. Higher dietary vitamin D intake was associated with a lower risk of developing AD among older women.

4.4.5.3 Prospective studies

Two prospective randomised controlled trials investigated if vitamin D treatment improved cognitive scores in patients with mild-moderate dementia with conflicting results

(Gangwar et al., 2015; Stein et al., 2011b). The first study included a population of outpatient participants receiving the same regimen of medical treatment for their dementia, supplementing one group with additional vitamin D. A total of 80 participants were randomly divided into treatment (n=40) and control (n=40) groups. All participants had low cognitive scores at baseline, determined by scores <24 on the mini mental state examination (MMSE), and were vitamin D deficient with serum vitamin D levels of <30 ng/ml before the study began. The treatment regime included oral granules of vitamin D3 (4000IU/d). After 6 months of supplementation, MMSE scores were significantly higher in the treatment group compared to controls, supporting that vitamin D3 has a beneficial effect on cognitive functions in an elderly population. The second trial was shorter in duration and supplemented with oral capsules of vitamin D2. All participants received low doses of vitamin D2 (1000IU/d) for a period of 8 weeks, then were randomly allocated to high dose (6000IU/d) vitamin D2 or placebo for a subsequent 8 weeks (Stein et al., 2011b). All participants had mild-moderate AD determined by MMSE scores between 12 and 24 recorded by an occupational therapist. At the conclusion of the study, no significant differences were observed in MMSE scores between treatment groups. Therefore, it was concluded that high dose has no protective benefit compared to low dose vitamin D (Stein et al., 2011b).

In summary, there again were few studies available to comprehensively reflect on a potential neuroprotective role for vitamin D in AD and if this was dependent on route of administration. The included studies predominately suggest that AD is associated with low serum vitamin D levels and increasing serum vitamin D supplementation may improve disease outcome; however, further research is required to better understand this relationship. No studies investigated the impact of sun exposure on neurological outcomes in AD.

4.4.6 Amyotrophic lateral sclerosis

Table 10 lists the study characteristics of all identified studies from the described search specific to ALS. A total of five studies were included in the final analysis consisting of pre-clinical studies (n=4) and a longitudinal cohort study (n=1).

4.4.6.1 Pre-clinical studies

All four pre-clinical studies tested potential therapeutic benefits of vitamin D treatment for ALS by manipulating vitamin D in the diet of G93A transgenic mice with a mutation in the SOD1 gene, producing the ALS phenotype. Of the four studies, one study demonstrated that adequate vitamin D3 levels had a protective effect in ALS (Moghimi et al., 2015), another study, using the same study design, reported mixed results, showing that vitamin D deficiency decreases early disease severity and delays disease onset, but reduces performance in functional outcomes following disease onset (Solomon et al., 2011). The final two studies found no significant differences between high vitamin D diets when compared with adequate intake control animals (Gianforcaro et al., 2013; Gianforcaro and Hamadeh, 2012).

The first two studies restricted vitamin D intake by reducing the level of vitamin D within chow to 0.025 IU/g compared to control animals receiving adequate levels of vitamin D at 1IU/g (Solomon et al., 2011; Moghimi et al., 2015). In one study, the spinal cords of both male and female mice were analysed for pathological markers of oxidative stress and inflammation as indicators of disease severity (Moghimi et al., 2015). Vitamin D3 deficiency exacerbated disease pathology through mechanisms that appeared to be sex-specific, as deficient males were affected more than deficient females (Moghimi et al., 2015). However, the second study, following the same restricted vitamin D diet showed a vitamin D deficiency reduced severity of disease and delayed onset, however, functional

performance in these animals was still impaired (Solomon et al., 2011). Indicting vitamin D deficiency may differentially affect functional and disease outcomes which was hypothesised to result from a gradual decrease in circulating pre-vitamin in the deficient animals, with a concomitant increase in circulating levels of active vitamin D (Solomon et al., 2011).

The remaining two pre-clinical studies were performed by the same research group and examined enriched vitamin D in the diet using increasing levels of vitamin D3 supplementation (Gianforcaro et al., 2013; Gianforcaro and Hamadeh, 2012). Neither study showed statistically significant differences in the functional outcome measures between groups. The first study in 2012 used a 10-fold increase in vitamin D, supplementing chow to 10 IU/g. Results were suggestive that vitamin D3 supplementation attenuated the decline in functional capacity, as indicated by impaired paw grip endurance and motor performance in the mouse model of ALS. However, this did not reach statistically significant levels. In the follow up 2013 study, vitamin D3 levels were enriched with a 50-fold increase to 50IU/g, and this attenuated the decline in paw grip endurance, but did not influence age of onset or disease severity. Suggestive of a tissue specific response to vitamin D in the CNS compared to skeletal muscles (Gianforcaro et al., 2013).

4.4.6.2 Longitudinal cohort study

The clinical study was a longitudinal cohort study investigating if vitamin D3 supplementation for a period of 9 months reduced scores on the Amyotrophic Lateral Sclerosis Functional Rating Scale (ALSFRS-R) in ALS patients (Karam et al., 2013). The study population included 37 ALS patients with a median time since symptom onset of 61 months. Participants were divided into patients receiving 2000 IU/d vitamin D3 supplementation (n=20) and participants without supplementation (n=17). At the end of the 9-month follow up period, the decline in the ALSFRS-R score was significantly smaller in

ALS patients receiving vitamin D3 compared to those who were not supplemented with vitamin D3. The result of this clinical trial indicate that increased vitamin D supplementation and increased vitamin D levels benefit ALS patients.

In summary, very few studies have been published addressing vitamin D levels in the onset and progression of ALS. The pre-clinical data suggest that vitamin D depletion produces poorer disease outcomes, although the results have been mixed. Additionally, there were no increased protective effects from increasing vitamin D intake above adequate levels in the animal studies reviewed. However, the clinical study supplemented above adequate intake levels and reported no adverse events. No studies relating to ALS investigated the effect of UV derived vitamin D.

Table 4.2. Multiple sclerosis summary results: Preclinical

Study	Study Design	Source of Vitamin D	Aim	Protective Benefit	Primary Outcomes
Tarbali et al. 2016	Toxin-induced rat model *2µl LPC in hippocampus* • N=7 LPC only • N=7 5ug/kg VD3 IP at days 7 & 21 post lesion • N=7 5ug/kg VD3 IP in sesame oil, no LPC • N=7 sham (150µl sesame oil IP, no LPC) • N=7 control (saline injection in CA1) • N=7 control (no surgery & no treatment)	IP vitamin D3	Assess the effect of vitamin D3 on behavioural processes in the toxin induced demyelination model within the CA1 area of the hippocampus of the rat.	✓	Vitamin D3 treatment is able to reduce spatial learning and memory deficits, through its antioxidant effects in an experimental model of MS.
Adzemovic et al. 2013*	EAE rat model *VD enriched chow with 2 & 10 IU/gram before EAE* • N=6-16 pre/early post-natal (3wks pre-lesion) • N=6-16 juvenile rats (3wks pre-lesion) • N=6-16 adult rats (8wks pre-lesion) • N=6-16 matched control (standard chow)	Oral vitamin D3	Investigate if there is age-depended efficacy of VD to prevent/ameliorate neuroinflammation and elucidate the most appropriate timing for treatment.	✓	VD ameliorates clinical symptoms in juvenile/ adolescence rats, but not in adult rats or rats treated during pre- and early post-natal development ($P<0.5$).
Garcion et al. 2003*	EAE rat model • N=39 0.5ug/kg VD3 IP on days 11, 13 with 0.1ug/kg VD3 (depleted chow) on days 19, 21 & 23 • N=26 matched control (vehicle) • N=9 0.05ug/kg MC2188 daily IP on days 10-23 • N=9 0.02ug/kg MC2188 daily IP on days 10-23 • N=9 control (no MC1288 from day 10-23)	IP vitamin D3/ deprivation	Determine if active vitamin D compounds (1,25D3 and MC1288) can achieve curative treatment in rat model of EAE.	✓	Beneficial improvement on clinical symptoms, cellular and molecular event within the CNS from VD3 treatment. Low doses of MC1288 led to non-significant differences on the first paralytic attack but displayed inhibitory benefit on incidence and amplitude of second paralytic attack.
Nashold et al. 2000	EAE mouse model *Disease severity score of 2.5-3* • N=7 100ng VD3 in soybean oil IP & 50ng/d VD3 chow • N=6 control (IP soybean oil and standard chow)	Oral/ IP vitamin D3	Investigate if VD3 treatment promotes Th2 cell differentiation inhibiting EAE.	✓	VD3 decreases paralysis in severe EAE ($P=0.03$). No difference between groups using flow cytometric analysis of CD4+/CD8+ T cells, B cells or macrophages in draining lymph nodes or spinal cord.

Table 4.2. Multiple sclerosis summary results: Preclinical *(continued)*

Study	Study Design	Source of Vitamin D	Aim	Protective Benefit	Primary Outcomes
Nashold et al. 2013	EAE Mouse model *Disease severity score 1.5 ± 0.5* • N=11-12 females fed +/- 0.33ug/day VD3 chow • N=11-12 males fed +/- 0.33ug/day VD3 chow *Disease severity score 2.3 ± 0.3* • N=6-8 200ng/ 0.1mL calcitriol in oil IP • N=6-8 weekly 200ng/ 0.1mL in oil IP for x3 • N=6-8 control (matched vehicle for all groups) • N=13 200ng calcitriol IP with 5µg VD3 via gavage and +VD diet (1ug/d) • N=15 control (matched placebos, -VD diet)	Oral/ IP vitamin D3	Investigate if vitamin D3, and the vitamin D3 hormone, calcitriol as treatments in EAE, a rodent model of MS.	✓	One calcitriol dose (active VD3) induced a transient remission in chow fed mice with EAE. Weekly calcitriol treatment induced sustained remission and prevented disability progression. One calcitriol dose + VD3 supplementation reduced pathology and improved ambulation.
Spach et al. 2005	EAE mouse model *VD enriched chow with 1 and 5ug/d 4 weeks prior to EAE 30 days post VD, females had oophorectomy* • N=15-28 female +/- VD • N=15-28 male +/- VD • N=12-28 matched control (standard chow) • N=15-28 female OVX +VD. • N=15-28 female sham +VD	Oral vitamin D3	Using the EAE model to investigate VD intake on MS risk.	✓	VD3 inhibited EAE in female mice but not male nice ($P<0.05$). The effect was lost in ovariectomized females.
Spach et al. 2006	EAE mouse model *Enriched chow: 0 or 1ng/d 4wks before EAE* • N=5 +/- VD, male and female • N=5 IL-10 gene KO, male and female • N=5 IL-10 receptor KO, male and female *Enriched chow: 50ng/d (females) and 100ng/d (males) 4wks before EAE* • N=5 +/- VD, male and female • N=5 IL-10 gene KO, male and female	Oral vitamin D3	Investigate how vitamin D3 metabolites inhibit EAE induction in wild type and knock out mice.	✓	The +D diet reduced incidence, peak clinical score and cumulative disease index in female but not male EAE mice. Both male and female mice fed biologically active VD had a lower incidence of EAE, decreased mortality and lower peak severity and cumulative disease index. However, +D protection was not observed in KO mice.
Pedersen et al. 2007	EAE rat model *Disease severity score of 2.5-3* • N=8 200ng IP VD3 in soybean oil and 100ng/d VD3 enriched chow • N=12 control (IP soybean oil and standard chow)	Oral/ IP vitamin D3	Investigate the anti-inflammatory functions of VD3 in an EAE model.	✓	At 24 hr post treatment transcripts of CXCL10, CCL2, and CCL3 were significantly less in the VD group compared to controls ($P<0.02$).

Table 4.2. Multiple sclerosis summary results: Preclinical *(continued)*

Study	Study Design	Source of Vitamin D	Aim	Protective Benefit	Primary Outcomes
Sloka et al. 2015	EAE mouse model *18 days post lesion* • N=4 Histology control of peak severity • N=16 Bi-daily IP 100ng VD3 in DSMO x 56 days • N=19 Control Bi-daily IP DSM x 56 days	Oral/ IP vitamin D3	Investigate if VD3 treatment can reduce axonal injury in EAE.	✓	VD3 caused significant improvements in clinical score from day 26 compared to controls ($P<0.05$). VD3 treatment prevented axonal loss relative to controls ($P<0.05$).
Soleimani et al. 2013	EAE mouse model *Disease severity score of 1* • N=6 IP 5mg/kg VD3 in sesame oil • N=6 control (IP of sesame oil) • N=6 control (EAE treatment only) • N=6 control (no intervention)	IP vitamin D3	Investigate the effect of VD treatment on TH1/Th2 ratio in EAE treated mice.	✓	Decreased clinical symptoms in VD group compared to control animals ($P<0.05$). Levels of TNF-α but not IL-10 significant decreased following VD3 administration.
Nataf et al. 1996	EAE rat model • N=7 EAE and 5µg/kg IP VD3 • N=5 EAE and 6µg/kg IP pre-VD3 • N=7 EAE and IP vehicle • N=15 EAE and no VD • N=3 control (no EAE and no VD3) • N=4 control (no EAE and 5µg/kg IP VD3)	IP vitamin D3	Evaluate the ability of VD3 to inhibit chronic relapsing EAE, when administered after the beginning of clinical signs.	✓	Significant clinical improvements in VD3 treated EAE rats ($P<0.05$). The effect was accompanied by marked inhibition of CD4 antigen expression in the CNS.
Farias et al. 2013	EAE Rat model *VD administration following EAE for 20 days* • N=15 15ug VD3 via gavage • N=15 10ug VD3 via gavage • N=15 5ug VD3 via gavage • N=15 2.5ug VD3 via gavage • N=15 15ug VD3 via IP injection • N=3 control (EAE but no VD)	Gavage/ IP vitamin D3	Investigate the effects of vitamin D treatment and the induction of tolerogenic activity of dendritic cells in the EAE model.	✓ / ✗	In vivo administration of VD3 significantly reduced EAE severity in treatment groups that received 10 and 15ug of VD via gavage. However, no effect on clinical evolution was observed in groups that were given lower gavage concentrations of 5 and 2.5ug or when the IP route was used.
Wang et al. 2012	EAE mouse model • N=10 VDR KO • N=9-11 25-hydroxylase KO • N=9-11 1α-hydroxylase KO • N=9-11 VD-deficient • N=12 control (matched WT animals)	Vitamin D receptor KO	To determine how the VDR relates to the developments of EAE by using different knock out strains and dietary restriction of VD.	✗	EAE development was suppressed in VD receptor KO mice and partially suppressed in mice with insufficient VD ($P<0.05$). Elimination of both key hydroxylases neither inhibited nor enhanced the development of EAE.

Table 4.2. Multiple sclerosis summary results: Preclinical *(continued)*

Study	Study Design	Source of Vitamin D	Aim	Protective Benefit	Primary Outcomes
Becklund et al. 2010	EAE mouse model *Pre-treatment: UV for 7d before EAE* • N=11-12 2.5 or 5kJ/m^2 • N=7 control: No UV exposure *Continuous: UV 7d before EAE +25-30d post EAE* • N=11 2.5 kJ/m^2 every second day • N=11 2.5kJ/m^2 every third day • N=11 control: No UV exposure *VD enriched diet: 10d before EAE +25-30d post EAE* • N=17 VD 10µg/kg/day 25(OH)D3 • N=17 VD 500µg/kg/day 25(OH)D3 • N=17 VD 1000µg/kg/day 25(OH)D3 • N=17 VD 2.5µg/kg/day 1'25(OH)D3 • N=15 control (matched vehicle)	UVR and oral vitamin D	Investigate the ability of UVR to suppress disease severity in EAE.	✗	UV-B pre-treatment did not suppress EAE and caused a slight increase in in serum VD. Continuous UV-B treatment suppresses EAE and causes transient increase in serum VD ($P<0.05$ compared to control). 25(OH)D3 only modestly suppressed EAE at doses that cause severe hypercalcemia.
Wang et al. 2013	EAE Mouse Model Daily UV 7d before EAE and continued for 30 days • N=12 2.5, 5 and 10 kJ/m^2 BB-UV-B • N=12 2.5 and 5 kJ/m^{2}NB-UV-B • N=12 2.5 and 10 kJ/m^2 BB-UV-A • N=12 10 kJ/m^2 UV-A-1 • N=12 control (no UV exposure)	UVR	Investigate the protective effects of UVR at different wavelengths on EAE induced mice.	✗	The results demonstrate that NB-UV-B is largely responsible for light-induced suppression of EAE and its effect is not via production of VD.
Wang et al. 2015	EAE mouse model Daily UV 7 days before EAE and continued for 30 days • N=8 10kj/m^2 BB-UV-B • N=12 control (no UV exposure	UVR	Investigate the mechanisms underlying UV-B suppression of EAE.	✗	Results suggest that UV-B irradiation suppresses EAE by selectively blocking the infiltration and binding of inflammatory cells into CNS. Likely mediated by selective inhibition of CCL5 in the CNS and assisted by a systemic increase in IL-10.

BB-UV-A, broadband UV-A (300-400nm); BB-UV-B, broad band UV-B (280-330nm); CNS, central nervous system; DSMO, Dimethyl sulfoxide; EAE, experimental autoimmune encephalomyelitis; IL-10, interleukin 10; IP, intraperitoneal; KO, knock out; LPC, lysophosphatidyl choline; NB-UV-B, narrow band UV-B (300-315nm); UV-A-1; ultraviolet A-1(340-400nm); TNF-α, tumour necrosis factor- α; UV, ultraviolet; VD, vitamin D; VDR, vitamin D receptor; WT, wild type; 25(OH)D, 25-hydroxyvitamin; funded by organizations that may have was funded by a grant from Biogen; ✓, study outcomes were supportive; ✗, study outcomes were contradictory; *Financial disclosure statement reporting funding grants from organizations that may create potential bias

Table 4.3: Multiple sclerosis summary results: Clinical: Retrospective case-control

Study	Study Design	Source of vitamin D	Aim	Protective Benefit	Primary Outcomes
Bjornevik et al. 2014*	Self-report questionnaire: Sun habits during childhood and adolescents • N=1660 MS patients • N=3050 controls	Past sun exposure	Estimate the association between MS and measures of sun exposure in specific age period in Norway and Italy.	N/A Protective effect of UV	A significant association between infrequent summer outdoor activity and increased MS risk was found in Norway and in Italy.
Kampman et al. 2007	Self-report questionnaire: Sun habits and diet at ages 6-10, 11-15, and 16-20 years • N=152 MS patients • N=402 controls	Past sun exposure and diet	Investigate if sunlight exposure or VD-related dietary factors in childhood and adolescents are associated with MS risk.	N/A Protective effect of UV	Increased outdoor activities during summer in early life were associated with a decreased risk of MS.
Dalmay et al. 2010	Interview-questionnaire: Sun habits before age 15 • N=193 MS patients • N=358 controls	Past sun exposure	Investigate the protective role of childhood UV exposure in patients with MS in Cuba, Martinique and Sicily.	N/A Protective effect of UV	Sun exposure before age 15 was associated with reduced MS risk, with evidence of dose response.
Islam et al. 2007	Interview questionnaire: Childhood sun exposure • N= 197 pairs MS disease-discordant MZ twins	Past sun exposure	Investigated the role of childhood sun exposure on the risk of MS after controlling for genetic susceptibility.	N/A Protective effect of UV	Protective effect of sun exposure on MS risk among MZ twins. For each unit increase in sun exposure index, the relative risk of MS decreased by 25%.
Van Der Mei et al. 2003	Interview questionnaire: Sun habits from childhood to adolescents • N=86 MS patients • N= 272 controls	Past sun exposure	Examine whether past high sun exposure is associated with a reduced risk of multiple sclerosis in Tasmania.	N/A Protective effect of UV	Higher sun exposure during childhood and early adolescence was associated with a reduced risk of multiple sclerosis (adjusted OR 0.31, (95% CI 0.16-0.59).
Baarnhielm et al. 2012*	Self-report questionnaire: Sun habits during the last 5 years • N=1013 MS patients • N=1194 controls	Past sun exposure	Examine the association between past UV exposure and MS, vitamin D levels inclusion of the study and occurrence with MS.	N/A Protective effect of UV	Participants with low UV exposure significantly increased risk of MS compared to those who reported high UV exposure (OR 2,2, 95% CI 1.5, 3.3). Results suggest UV may exert effects independently from vitamin D.
Espinosa-Ramírez et al. 2014	Interview questionnaire: Sun habits during adolescents and the last 5 years. • N=83 MS patients • N=166 controls	Past/present sun exposure	Analysed past and current sun exposure in MS as compared with matched controls in Mexico.	N/A NO Protective effect of UV	No association between sunlight exposure and the risk of developing MS through UVR.

N/A, not available (data relates to UV exposure only); MS, multiple sclerosis; MZ, monozygotic; UV, ultraviolet; VD, vitamin D; *Financial disclosure statement reporting funding grants from organisations that may create potential bias

Table 4.4: Multiple sclerosis summary results: Clinical: Cross-sectional

Study	Study Design	Source of Vitamin D	Aim	Protective Benefit	Primary Outcomes
Jelinek et al. 2015+	Self-report data of lifestyle from MS patients from 57 countries combined with environmental factors such as geographic location (N=2301).	Lifestyle factors	Investigate the associate of lifestyle factors with quality of life and disease outcomes of an international MS cohort	✓	Association between latitude, deliberate sun exposure and VD supplementation and health outcomes in which VD is likely to have a key role (P=.014).
McDowell et. al 2011	Self-report data on histories of residential locations, sun exposure and intake of VD were used to estimate VD-related exposures in MS patients compared to residential solar estimates (N=1328).	Lifestyle factors	Investigate if VD intake and sun exposure during childhood and adolescents are associated with reduced risk of MS.	✓	Association between VD-related exposures during childhood and early adolescence and the timing of MS symptom onset and supports VD as a potential modulator of the clinical course of this disease.
McDowell et al. 2011	Self-report questionnaire from ex veterans on MS milestones using PDDS scale, level of sun exposure from age 6 until disease onset and VD dietary/ supplementary intake (N=219).	Lifestyle factors	Investigate VD-related exposures from childhood to disease onset and their association with MS progression	✓	Low average sun exposure before disease during fall/winter was associated with an increased risk of progressing to a PDDS score of 8 (HR: 2.13, 95% CI: 1.20-3.78) whereas use of cod liver oil during childhood and adolescences was associated with reduced risk (HR: 0.44, 95% CI: 0.20–0.96).
Mandia et al. 2014	Three questionnaires on lifetime smoking, diet, and sun exposure in the past three years and current levels of serum VD from a sample of MS patients (N=131).	Lifestyle factors	Examine whether environmental factors play a key role in the onset of MS including data on smoking habits, sunlight exposure and diet	✓	Adequate VD levels and frequent sun exposure are associated with mild MS, therefore suggesting that these environmental factors could play a role in MS pathogenesis.
Van Der Mei et al. 2003	Interview questionnaire of life style factors relating to lifetime VD exposure compared to serum VD in MS cases (N=136) and matched controls (N=272).	Lifestyle factors	Examine the prevalence and determinant of VD insufficiency in a population-based sample of MS cases and controls in Tasmania, Australia.	✓	Strong associations between disability, sun exposure and VD status indicate that reduced exposure to the sun, related to higher disability, may contribute to the high prevalence of VD insufficiency found in this population-based MS study.
D'Hooghe et al. 2012	Self-report questionnaire on MS characteristics, level of sun exposure (N=894), work place and skin type characteristics (N=1372).	Lifestyle factors	Investigate if sun exposure and phenotypic skin characteristics are associated with progression of disability in MS.	✓	Relapsing and progressive MS had separate patterns of association with sun exposure. Increased sun exposure in the past 10 years decreased the risk of reaching EDSS 6 in relapsing MS. Whereas, higher sun sensitivity was associated with increased hazard of reaching EDSS 6 in progressive MS.
Lucas et al. 2011*	Self-report sun exposure by life stage compared to objective measures of actinic damage, serum VD and skin phenotype in MS patients (N=282) and controls (N=395 controls).	Lifestyle factors	Examine the extent to which the latitudinal gradient and whether past and recent sun exposure and VD status contributes to the incidence of first demyelination events across Australia	✓ / ✗	Higher recent or lifetime sun exposure was associated with reduced risk of first demyelinating event. Recent sun exposure and VD levels independently contributed to reduced risk of a demyelinating event

Table 4.4: Multiple sclerosis summary results: Clinical: Cross-sectional *(continued)*

Study	Study Design	Source of Vitamin D	Aim	Protective Benefit	Primary Outcomes
Hejazi et al. 2014	Self-report questionnaires on sun exposure, food frequency intake and vitamin supplementation in MS cases (N=37) compared to control cases (N=37).	Lifestyle factors	Compare serum levels of 25(OH)D and total antioxidant status (TAS), dietary intake of antioxidant vitamins and vitamin D sources in MS patients to healthy controls.	✗	No difference between vitamin D and TAS between groups. Dietary intake of vitamin D and antioxidant vitamins also showed no difference between groups.
Gelfand et al. 2011*	Serum samples from African American with MS (N=339) compared to matched controls (N=342).	Lifestyle factors	Evaluate if VD is associated with MS status and disease severity in African Americans	✗	No association between VD status and disease severity (P=0.57). Levels of 25(OH)D were lower in African Americans with MS compared to controls, an observation primarily explained by differences in climate and geography.

CI, Confidence Interval; HR, Hazard risk; EDSS, expanded disease severity scale; MS, Multiple sclerosis; PDDS, Patient Determined Disability Scale; UV, ultraviolet radiation; VD, vitamin D; 25(OH)D, 25-hydroxyvitamin; ✓, study outcomes were supportive; ✗, study outcomes were contradictory +Disclosure statement reporting potential competing interests, *Financial disclosure statement reporting funding grants from organisations that may create potential bias

Table 4.5: Multiple sclerosis summary results: Clinical: Prospective

Study	Study Design	Source of Vitamin D	Aim	Protective Benefit	Primary Outcomes
Farsani et al. 2015	Clinical trial: • N=32 50,000 IU/week of VD • N=32 matched controls (serum only)	Intra-muscular injection	Evaluate the effect of vitamin D supplementation on the mRNA expression of IL-10 and TGF-B1 genes in MS patients and their correlation with clinical features.	✓	After 8 weeks of treatment with vitamin D, MS patients had a significant reduction in the mean EDSS scores of the population.
Derakh-shandi et al. 2013	Randomized controlled trial *12 months: double blind, ON patients pre-MS* • N=13 50,000 IU/week of VD3 • N=11 placebo	Oral vitamin D3	Preventive effects of VD3 administration on the conversion of optic neuritis to MS	✓	Risk reduction was 68.4% for the treatment group (relative risk = 0.316, P=0.007).
Kragt et al. 2009	Longitudinal cohort study: *6 months follow up* • N=103 MS patients • N=110 healthy controls	Lifestyle factors	Examine the role of VD metabolites in MS by comparing serum VD levels with information on disease characteristics and environmental factors that may influence VD.	✓	Higher levels of circulating 25(OH)D are associated with a lower incidence of MS and MS-related disability in women. Every 10nmol/L increase of serum VD the odds of MS reduced by 19% (odds ratio 0.81; 95% CI: 0.69–0.95)
Simpson et al. 2010	Longitudinal cohort study: *3 years follow up* • N=145 MS patients	Lifestyle factors	Investigated if higher levels of serum VD were associated with decreased risk of relapse in MS patients.	✓	Increasing serum VD was associated lower relapse rate with each 10nmol/l increase in VD the hazard was reduced by 9% (95% CI, 3–15%) after adjusting for age and sex.
Loken-Amsrud et al. 2012*	Longitudinal cohort study *24 months follow up* • N=88 MS patients	Lifestyle factors	Examine the relationship of between vitamin D and disease activity in relapsing-remitting MS before and during interferon-β treatment.	✓ / ✗	Prior to IFN-β treatment increased vitamin D was associated with reduced odd for lesion development (14.1%). No association between vitamin D and disease activity was detected after initiation 0f IFN-β treatment.
Munger et al. 2004	Longitudinal cohort study *Nurses' Health Study (NHS) 1976 & 1989* • N=76 MS cases from NHS • N=97 MS cases from NHSII	Dietary vitamin D	Dietary vitamin D was examined in two large cohorts of women from baseline and updated every four years thereafter.	✓ / ✗	Women who supplemented vitamin D had a 40% lower risk of developing MS than women who did not supplement. However, there was no association between vitamin D intake form food and the risk of MS
Achiron etal. 2015	Randomized controlled trial *6 months: double blind in MS patients* • N=80 patients given 1mcg/day VD3 • N=78 placebo	Oral vitamin D3	Evaluate the effect of VD analogue, alfacalcidol, on MS-related fatigue.	✓ / ✗	Decreased mean relative FIS score compared to placebo (41.6% vs. –27.4%, P=0.007, respectively). No significant differences in EDSS observed during the study.

Table 4.5: Multiple sclerosis summary results: Clinical: Prospective *(continued)*

Study	Study Design	Source of Vitamin D	Aim	Protective Benefit	Primary Outcomes
Soilu-Hänninen et al. 2012	Randomized controlled trial *12 months: double blind in MS patients* • N=34 20000 IU/week of VD3 • N=32 placebo	Oral vitamin D3	Investigate the efficacy of vitamin D# as an add on therapy to IFN-β in patients with MS.	✓ / ✗	Vitamin D3 add on treatment reduces MRI disease activity however there were no significant difference in adverse events or in the annual relapse rate.
Stein et al. 2011*	Randomized controlled trial *6 months: double blind in MS patients* • N=11 12000 IU/d high dose VD • N=12 1000 IU/d low dose VD	Oral vitamin D3	Evaluate the effects of high dose VD2 compared to low dose supplementation in MS patients.	✗	No therapeutic advantage in RRMS for high-dose D2 over low-dose D2 supplementation. High-dose VD2 compared to low-dose supplementation was not effective in reducing MRI lesions in patients with RRMS.
Mosayebi et al. 2011	Randomized controlled trial *6 months: double blind in MS patients* • N=28 300,000 IU/month • N=34 control patients	Intra-muscular vitamin D3	Investigate the effects of short term VD3 therapy and its influence as an immuno-modulatory agent on MS susceptibility as well as on clinical course of the disease.	✗	No statistical difference between baseline EDSS scores and EDSS scores after 6 months of treatment. Cell proliferation in the VD treatment group were significantly lower than the control.
Shay-gannejad et al. 2012	Randomized controlled trial *12 months: double blind in MS patients* • N=25 escalating doses up to 0.5μg/day • N=25 given placebo	Oral vitamin D3	Assess the effects of low-dose VD in combination with current disease-modifying therapy in terms of their safety, tolerability, and relative efficacy in preventing the progression of RRMS.	✗	No significant effect on the EDSS score or relapse rate from adding VD to treatment regime.
Golan et al. 2013a	Randomized controlled trial *12 months: double blind in MS patients receiving IFN-β treatment* • N=21 4370 IU/day VD3 • N=24 800 IU/day VD3	Oral vitamin D3	Investigate if VD supplementation ameliorates IFN-β induced flu-like symptoms in patients with MS.	✗	No significant change in FLS between groups. No significant differences in relapse rate, EDSS, quality of life or serum levels of IL-10 and IFN-γ. IL-17 levels were significantly increased in the low dose group only.
Kampman et al. 2012	Randomized controlled trial *18 months: double blind* • N=35 20,000 IU/week VD3 • N=33 placebo	Oral vitamin D3	Assess if VD supplementation effects bone mineral density MS patients, as well as, explore if VD improves disease outcomes.	✗	Supplementation did not result in beneficial effects on the measured multiple sclerosis-related outcomes.
Knippenberg et al. 2014	Longitudinal study *2-3 years follow up* • N=98 MS patients	Lifestyle factors	Examine the association between personal sun exposure and serum 25(OH)D, and depression, anxiety, fatigue and cognition.	✗	Higher reported sun exposure, rather than serum VD levels were associated with less depressive symptoms and fatigue. No correlation of serum VD/reported sun & cognition.

CI, confidence interval; EDSS, expanded disability status scale; IFN-β, interferon-β; IU, international units; FIS, Fatigue Impact Scale; FLS, flu-like symptoms; ON, optic neuritis; NHS, Nurses' health study; RRMS, relapsing-remitting multiple sclerosis; ✓, study outcomes were supportive, ✗, study outcomes were contradictory; *Financial disclosure statement reporting funding grants from organisations that may create potential bias

Table 4.6: Parkinson's disease summary results: Preclinical

Study	Study Design	Source of Vitamin D	Aim	Protective Benefit	Primary Outcomes
Cass et al. 2014	6-OHDA (12µg) rat model *4 weeks post lesion rats injected daily for 8d* • N=8 0.3µg/kg/day VD3 • N=8 1µg/kg/day VD3 • N=8 control (matched vehicle)	Subcutaneous vitamin D3	Examine the ability of calcitriol to promote restoration of extracellular DA levels and tissue content of DA in 6-OHDA treated animals.	✓	Increased potassium and amphetamine evoked overflow of striatal DA (P<0.05). Increased striatal and nigral tissue levels of DA, on the lesioned side of the brain (P<0,05).
Kim et al. 2006	6-OHDA (8µg) rat & MPTP (20mg/kg) mouse models *Pre-treatment 7days before lesion* • N=12 1µg/ml/kg/day VD3 (6-OHDA) • N=28 1µg/ml/kg/day VD3 (MPTP) • N=12/28 control (matched vehicle)	IP vitamin D3	Investigative the neuroprotective effects of VD3 against pre-clinical toxicity modes of PD.	✓	Decreased nigrostriatal degeneration with VD3 pre-treatment in both the 6-OHDA and MPTP models of PD (P<0.05).
Sanchez et al. 2009	6-OHDA (8µg) lesion model: *Daily doses of 1µg/ml/kg VD3* • N=30 7 days pre- 6-OHDA • N=30 7 days pre- and 28d post-6-OHDA • N=30 control (sham lesion and VD3) • N=30 control (6-OHDA lesion and no VD3)	IP vitamin D3	Investigate if VD3 treatment enhances GDNF mRNA expression.	✓	Treatment with VD3 before and after 6-OHDA injection partially restored TH expression in substantia nigra (P<0.001).
Wang et al. 2001	6-OHDA (8µg) lesion model: *Daily doses of 1µg/ml/kg VD3* • N=16 7 day pre-6-OHD • N=16 controls (matched vehicle)	IP vitamin D3	Investigate if administration of VD3 in vivo and in vitro would protect against 6-OHDA-induced DA neuron injury.	✓	D3 pre-treatment protected against 6-OHDA-mediated depletion of DA and its metabolites in substantia nigra (P <0.05).
Smith et al. 2006	6-OHDA (100µg) lesion model *Daily doses of 1µg/ml/kg VD3* • N=7-11 pre-6OHD • N=7-11 7 day pre- and 28 days post-6-OHD • N=7-11 controls (matched vehicle)	IP or subcutaneous vitamin D3	Examine the neuroprotective effects of short and long VD3 treatment in a model of early stage PD.	✓ / ✗	No protective effect of VD3 in short term group. Increased protective effects in levels and release of dopamine with long term VD treatment (P<0.01).
Dean et al. 2012	MPTP (15mg/kg) • N=8 VD deficient chow 6 weeks pre-lesion • N=8 control (standard chow)	Vitamin D restriction	Investigate the effect of VD deletion on the neuronal susceptibility to MPTP.	✗	Short term 25(OH)D depletion over the course of a few weeks does not render dopamine neurons more susceptible to the neurotoxin, MPTP.

DA, dopamine; GDNF, glia derived neurotrophic factor; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; VD, vitamin D; 6-OHDA, 6-hydoxydoamine; 25(OH)D, 25-hydroxyvitamin; ✓, study outcomes were supportive; ✗, study outcomes were contradictory

Table 4.7: Parkinson's disease summary results: Clinical

Study	Study Design	Source of vitamin D	Aim	Protective Benefit	Primary Outcomes
Suzuki et al. 2013	Randomised control trial *12 months double blind* • N=1200 IU/day • N=58 pacebo	Oral vitamin D3	Assess if VD3 supplementation inhibits progression of PD based on patient VDR groups.	✓ / ✗	Stabilized severity of PD in patients with FokI CT (P=0.02) and FokI TT (P=0.009) genotypes for study period but not inpatients with the FokI CC genotype.
Wang et al 2016	Cross-sectional case control • N=201 cases • N=199 controls	Circulating levels of pre-vitamin D	Measure serum VD, sunlight exposure, VD intake and evaluate the risk of PD.	✓	Lower levels of serum VD and sunlight exposure are significantly associated with an increased risk for PD (P<0.05).
Wang et al. 2015	Cross-sectional case control • N=478 cases • N=431 controls	Circulating levels of VD2 and VD3	Measure VD2 and VD3 metabolites in PD patients to identify if source is associated with risk of PD.	✓	Both VD2 and VD3 levels are inversely associated with PD (P<0.02).
Zhu et al. 2014	Case control • N= 209 cases • N-610 controls	Past UVR	Evaluate the relationships between intake of VD, outdoor activities and the risk of PD.	✓	Outdoor activity and total VD intake were inversely associated with PD, however, outdoor activity is more significantly associated with decreased risk for PD (P=0.01).
Kwon et al. 2013	Case control • N=490 cases • N= 644 controls	Past UVR	Examine the relationship between PD and outdoor work.	✗	Occupational sunlight exposure and other correlates of outdoor work is not likely to have a substantial role in the aetiology of PD.
Miyake et al. 2011	Case control • N=250 cases • N=372 controls	Dietary vitamin D	Examine the relationship between the consumption of dairy products, calcium and VD on the risk of PD.	✗	No evident relationship observed between consumption of total dairy products and the risk of PD.

International units, IU; PD, Parkinson's disease; VDR, vitamin D receptor; ✓, study outcomes were supportive; ✗, study outcomes were contradictory

Table 4.8: Alzheimer's disease and dementia summary results: Preclinical

Study	Study Design	Source of Vitamin D	Aim	Protective Benefit	Primary Outcome
Bennet et al. 2013	APPswe/PS1dE9 Transgenic Mice *Daily VDM (0.12-0.2IU VD2) for 7 months* • N=12 VDM • N=13 vitamin D depleted chow • N=10 control (WT fed VDM) • N=11 control (WT fed -VD chow)	Oral vitamin D2	Investigate if vitamin D2-enriched button mushroom effects cognitive & pathological outcomes, compared with a VD-deficient base diet, in a transgenic mouse model.	✓	Significant benefits from VDM diet versus control feed was observed in 3/5 memory tests of WT animals but only 2/5 tests for Tg mice. VDM-Tg mice had significantly reduced amyloid plaque load and glial fibrillary acidic protein, and elevated interleukin-10 in the brain.
Landel et al. 2016	5XFAD Transgenic mice *Daily doses of 7500IU/kg or 1000IU/kg VD3* • N=16 fed high VD3 • N=14 fed low VD3 • N=11 control (WT fed high VD3) • N=10 control (WT fed low VD3)	Oral vitamin D3	Assess the potential therapeutic benefit and mechanism of action of a chronic vitamin D supplementation in an AD model.	✓	Five months of vitamin D3 supplementation decreased the quantity of plaques in three brain regions of Tg mice (frontal cortex, hippocampus and neocortex). At the end of supplementation period (8 months) there was significant decreases in performance observed in Tg mice compared to Vitamin D3 treated mice during both the Y maze and radial arm water tests.
Yu et al. 2011	AβPP-PS1 transgenic mice • N=5-10 standard chow • N=5-10 12IU/g VD3 enriched chow • N=5-10 0IU/g VD3 depleted • N=5-10 control (WT fed 12IU/gr VD3)	Oral vitamin D3	Investigate if Vitamin D3 supplementation affects amyloid plaque formation in APP transgenic mice.	✓	Vitamin D3 enriched diet correlates with a decrease in the number of amyloid plaques, a decrease in amyloid peptides, a decrease in inflammation, and an increase in NGF in the brains of APP mice.
Taghizadeh 2014	Intracerebroventricular injection of Aβ 1-42 • N=8 fed standard chow • N=8 fed 200ng/d VD3 enriched chow • N=8 0IU/gram VD3 depleted chow • N=8 control (vehicle & standard chow)	Oral vitamin D3	Assess if the different regimens of vitamin D underlie induction of LTP in the CA1 area of hippocampus in a rodent model of AD.	✓	Vitamin D3 deprivation promotes the weakened basic synaptic transmission in the Aβ-treated rats and intensifies inhibition of LTP in these animals. Vitamin D3 increased baseline activity and post-tetanus potentiation.
Taghizadeh et al. 2011	Intracerebroventricular injection of Aβ 1-42 • N=9 fed standard chow • N=10 1000ng VD3/100g chow • N=10 0IU/gram VD3 depleted chow • N=14 control (vehicle & standard chow)	Oral vitamin D3	Examine the effects of vitamin D supplementation or depletion on spatial performances in the AD animals.	✗	No significant difference in the spatial performance in Vitamin D treated rats compared to controls.

AD, Alzheimer's disease; APP, amyloid-protein precursor; Aβ, amyloid beta; Tg, transgenic mice; international units, IU; VD, vitamin D; VDM; vitamin D enriched mushrooms; LTP, long-term potentiation; NGF, nerve growth factor; TG, transgenic; WT, wild type; ✓, study outcomes were supportive, ✗, study outcomes were contradictory

Table 4.9: Alzheimer's disease and dementia summary results: Clinical

Study	Study Design	Source of Vitamin D	Aim	Protective Benefit	Primary Outcomes
Afzal et al. 2014	Cohort study *30 years follow up of participants with stored plasma from the Copenhagen Heart (beginning 1976-8)* • N=418 participants	Lifestyle factors	Assess if decreased plasma Vitamin D is associated with increased risk of AD and vascular dementia in the general population.	✓	During the 30 years of follow-up, 418 participants developed AD and 92 developed vascular dementia. Observed was an increasing risk of AD with decreasing levels of Vitamin D.
Annweiler et al. 2011*	Longitudinal Cohort Study *7 years follow up* • N=40 participants from the EPIDOS study	Lifestyle factors	Determine if VD measured at baseline could predict the onset of Non-Alzheimer's Dementia.	✓	Vitamin D deficiency at baseline was associated with the onset of Non-Alzheimer's dementia within 7 years.
Annweiler et al. 2012*	Longitudinal Cohort Study *7 years follow up* • N=498 participants from the EPIDOS study	Dietary Intake	Determine if dietary intake if vitamin D was an independent predictor of the onset of dementia among women.	✓	Baseline dietary was inversely associated with the onset of Alzheimer's disease within 7 years.
Gangwar et al. 2015	Randomized Controlled Trial *6 months follow up* • N=40 4000 IU/day VD3 • N=40 not given VD3	Oral vitamin D3	Evaluate VD supplementation on cognitive performance in subjects of cognitive impairment.	✓	Increased cognitive performance in subjects with senile dementia (P<0.02).
Stein et al. 2011	Randomized Controlled Trial *16 weeks duration: All patients on low dose VD (1000IU/day for duration of study)* • N=16 after 8 weeks high dose VD (6000IU/day) • N=16 after 8weeks given placebo	Oral vitamin D2	Examine the effects of high-dose vitamin D followed by nasal insulin on memory and disability in mild-moderate AD	✗	No significant benefit of high dose vitamin D on cognitive performance or disability in mild-moderate Alzheimer's disease.

AD, Alzheimer's disease; International units, IU; VD, vitamin D; ✓, study outcomes were supportive, ✗, study outcomes were contradictory *Disclosure statement regarding consultation roles of the authors

Table 4.10: Amyotrophic lateral sclerosis summary results

Preclinical					
Study	**Study Design**	**Source of Vitamin D**	**Aim**	**Protective Benefit**	**Primary Outcomes**
Moghimi et al. 2015	G93A mouse model of ALS • N=19 0.025 IU/g VD (reduced) • N=23 control (1 IU/g /day VD)	Vitamin D restriction	Investigate if VD deficiency effects apoptosis, oxidative damage, antioxidant capacity, inflammation, neurotrophic factor & neuron count in the spinal cord.	✓	Increased disease pathophysiology with VD deficiency in the spinal cord of G93A mice, the exact mechanisms are sex-specific ($p<0.05$).
Solomon et al. 2011	G93A mouse model of ALS • N=48 0.025 IU/g VD (reduced) • N=54 control (1 IU/g /day VD)	Vitamin D restriction	Examine VD deficiency on functional and disease outcomes, disease onset and lifespan.	✓ / ✗	Decreased PaGE and motor performance ($P<0.02$). Increased early disease severity and delays disease onset compared to a diet with adequate VD3 ($P=0.002$).
Gianforcaro et al. 2012	G93A mouse model of ALS • N=18 10 IU/g/day VD3 • N=14 control (1 IU/g /day VD)	Oral vitamin D3	Investigate high intake VD3 versus adequate intake on functional and disease severity outcomes in ALS.	✗	No significant differences in disease onset, disease progression, or lifespan between groups ($P=0.074$).
Gianforcaro et al. 2013	G93A mouse model of ALS • N=46 50 IU/g/day VD3 (high diet) • N=54 1 IU/g/day VD3 (adequate intake)	Oral vitamin D3	Investigate high intake VD3 versus adequate intake on functional and disease severity outcomes in ALS.	✗	High VD3 intake did not influence age at disease onset, hind limb paralysis or endpoint in the transgenic G93A mouse model of ALS. No significant difference between groups in functional tests when corrected for clinical scores.
Clinical					
Study	**Study Design**	**Source of Vitamin D**	**Aim**	**Protective Benefit**	**Primary Outcomes**
Karam et al. 2013*	Longitudinal Cohort Study *9 months follow up* N=20 ALS patients given 2000 IU/d VD3 N=17 ALS patients no supplementation	Oral vitamin D3	Measure serum VD levels in patients with ALS and reviewed the effect of VD supplementation.	✓	Decrease in ALSFRS-R score at 9 months of VD supplementation ($P=0.02$).

ALS, amyotrophic lateral sclerosis; ALSFRS-R, amyotrophic lateral sclerosis functional rating scale-revised; international units, IU; VD, vitamin D; ✓, study outcomes were supportive; ✗, study outcomes were contradictory *Disclosure statement stating first author served on the editorial board for Neurology

4.5 Discussion

In this systematic review, we explored the neuroprotective effects of vitamin D in neurodegenerative disorders. The focus was on comparing the effect of endogenously generated vitamin D, resulting from UV exposure, and different forms of exogenously derived vitamin D from synthetic supplementation. Additionally, we set out to identify whether vitamin D had a protective benefit on the *onset* or *development* of neurodegenerative diseases that was dependent on the route of administration.

The results indicate that current data supporting vitamin D as a neuroprotective agent is predominately based on pre-clinical and observational studies and that solid clinical evidence is lacking. The majority of clinical MS trials supplementing with vitamin D did not show significant difference/improvements between treatment groups, while the few clinical studies in PD, AD and ALS did show some positive results yet fewer prospective studies were identified. Unfortunately, very limited research was available to discuss the impact of vitamin D as a result of sun exposure on neurodegenerative diseases.

Another, key finding of this systematic review is that sun exposure may protect against MS, but that there is insufficient data to support the use of oral vitamin D supplementation as a *substitute* for UV sourced vitamin D in MS. The lack of published clinical research relating to PD, AD and ALS results in insufficient evidence to draw conclusions regarding oral vitamin D supplementation versus UV derived vitamin D for these diseases. Further, these data draw into question if vitamin D is the mediator of the neurodegenerative disease protective effects of sun exposure or whether it is associated with increased outdoor activity, which may drive independent neuroprotective benefits or an as of yet unknown UV-related neuroprotective effect.

Though the complex aetiology of MS, PD, AD, and ALS remain largely unknown, the pre-clinical models of these diseases support the hypotheses that insufficient vitamin D likely contributes to neurodegenerative disease. Of the 31 pre-clinical studies reviewed, 21 studies reported that vitamin D had a significant neuroprotective benefit in either delaying disease onset (Adzemovic et al., 2013; Spach and Hayes, 2005; Spach et al., 2006) or reducing disease symptoms (Bennett et al., 2013; Cass et al., 2014; Garcion et al., 2003; Kim et al., 2006; Landel et al., 2016; Nataf et al., 1996; Nashold et al., 2000; Nashold et al., 2013; Pedersen et al., 2007; Sanchez et al., 2009; Sloka et al., 2015; Soleimani et al., 2014; Solomon et al., 2011; Taghizadeh et al., 2014; Tarbali and Khezri, 2016; Wang et al., 2001; Yu et al., 2011). The pre-clinical evidence suggests that route of administration does not influence neuroprotective benefits from vitamin D. However, only the MS and PD pre-clinical studies incorporated both preventative and treatment interventions and looked at different age-related timing for intervention and treatment, whereas, the pre-clinical AD and ALS studies looked at the effects of vitamin D manipulation after disease onset. Further exploration of vitamin D's role in the onset of disease as well as in varying degrees of vitamin D deficiency are needed in these animal models to better understand potential protective benefits from vitamin D.

The pre-clinical literature for all disease types varied significantly in the way in which vitamin D was administered to animals and the vehicle substrates used. Independently, each of these factors are cause for significant variation in outcomes and this may in part contribute to the lack of clinical translation, as the bioavailability would vary between different delivery methods. Intraperitoneal injection was the most common form of administration in pre-clinical models, particularly in PD research, whereas oral supplementation was the predominate route in the clinical literature for all disease types.

The lack of consistency between groups means it is difficult to identify the best vitamin D intervention/treatment strategy to design clinical trials.

Therefore, whilst the majority of pre-clinical data reported a positive outcome attributed to vitamin D, this same positive association does not translate clinically, particularly for MS. Drawing into question whether animal models adequately reflect the human pathologies in this scenario or if there are other important considerations to be made when using pre-clinical models to investigate vitamin D in neurodegenerative diseases.

The observational data largely suggest that vitamin D may have a causative role in the pathogenesis of neurodegenerative diseases, with 15 of 21 studies highlighting that low serum vitamin D levels are associated with increased risk of neurodegenerative disease. Though, evidence from the observational research reported here, is not sufficient on its own to support the formal recommendation that a person's brain health would benefit from increased serum vitamin D concentrations. Rather, quality use of medicine mandates that any recommendations should be grounded in causal evidence from clinical trials (Thacher and Clarke, 2011). Observational studies, while useful for generating hypotheses, cannot prove causality and often involve many confounding factors (e.g. season, ageing, geographical latitude, body mass index (BMI), physical activity, diet, and smoking) that are difficult to fully control for and independently relate to vitamin D status and health outcomes (Antico et al., 2012). If vitamin D were causatively associated with increased risk of neurodegenerative disease, then results from oral supplementation would be expected to significantly improve disease outcomes. Yet, as the MS literature shows this is not the case, suggesting that disease development and progression is not solely dependent on vitamin D. The MS literature demonstrates that sun exposure is linked to decreased prevalence of MS although the seven clinical trials, with a total population size of 368

participants, showed oral vitamin D supplementation failed to provide neuroprotective benefit. This may indicate that, mechanistically, an additional factor, such as UVR, may be responsible for the benefits from sun exposure on disease outcomes (Knippenberg et al., 2014). The limited number of clinical studies available for PD, AD and ALS means it is not possible to determine from the systematically reviewed study results whether route of administration is a key factor in the neuroprotective benefits associated with vitamin D based on this review.

Information gained from the pre-clinical and observational studies should be used in designing clinical trials investigating a role for vitamin D in development and progression of a range of neurodegenerative diseases.

The current mechanistic model used to explain the relationship between increased UV exposure and decreased neurodegenerative disease is that UVR is critical for the production of vitamin D3. Sufficient vitamin D is thought to decrease the risk of neurological diseases by supressing pathological mechanisms through increases in anti-inflammatory properties (Mpandzou et al., 2016; Holick, 2011). However, the data presented here show synthetic vitamin D supplementation does not meet neuroprotective expectations for MS and more research is needed to support its use in the other diseases. It was expected that the different forms of vitamin D would provide a possible explanation as to why supplemented vitamin D may not adequately match UVR produced vitamin D. However, as most studies used vitamin D3 this is not likely a contributing factor. It is therefore possible that vitamin D may not be associated with neurodegenerative disease onset or disease progression but is merely an associative marker of sun exposure. As UV-B wavelengths contribute to the production of vitamin D, any increases in sun exposure will likely result in elevated serum vitamin D levels (Garcion et al., 1998). Yet, UVR has

other inflammatory properties that suppress the immune system through pathways independent of vitamin D, including the inhibition of antigen presenting cells, inducing suppressor T-cell populations, and altering inflammatory cytokine levels (Norval et al., 2008). Prospective studies need to consider that vitamin D may be an associative marker and ensure to design experiments that are able to separate vitamin D dependent and independent contributions from UVR.

There may be unintentional biases in the clinical studies, which cause an overestimation of the impact of sun exposure and vitamin D. For example, patients with less disability are more likely to have increased time in the sun participating in outdoor activities and therefore have increased levels of vitamin D when compared to those with greater disease severity**.** Moreover, increased physical activity correlates with increased time spent outdoors (Gray et al., 2015) and may be a confounding factor in the studies reviewed. People who are more physically active and spend more time outside consistently present with improved health outcomes (Bauman et al., 2016; Mead, 2008). Independently, physical activity in an important preventative factor against disease with supporting evidence for both PD and AD (Paillard et al., 2015). Diet is another potentially cofounding variable that may have contributed to unintentional bias. People who eat meat and fish have higher plasma concentrations of circulating vitamin D than those who follow a vegetarian or vegan diet (Crowe et al., 2011).

Regardless of any statistical adjustment for potential interactions between co variables including sun exposure, physical activity and vitamin D status residual confounds may exist in the observational studies reported here. Adequate control variables of factors that may impact disease severity and vitamin D status are needed when designing clinical trials. Including physical activity, mood, diet and actual time outdoors/ UV index, which as the present time are predominately dependent on the patient's subjective evaluation and

may not be reliable. Future prospective studies would have the potential to overcome this through sunlight detection sensors and the use of activity monitors.

Definitions of vitamin D insufficiency/deficiency determined by serum levels of 25(OH)D also varied across studies. As such, it is evident that there is no consensus on the appropriate supplemented vitamin D dose or required level. Nor is there an apparent dose-to-target plasma concentration of 25(OH)D relationship employed. Amongst the literature reviewed here, a variety of oral vitamin D doses were used, ranging tenfold, from 1200 IU to 12,000 IU per day. The majority (9/12) of prospective clinical studies included in this systematic review supplemented vitamin D doses above the current recommendation of 800 IU per day (Institute of Medicine Standing Committee on the Scientific Evaluation of Dietary Reference, 1997), and often for only short periods of follow up. Another major problem with clinical trials regarding nutrients, such as vitamin D, is that they follow guidelines that have been designed for pharmaceutical drugs without taking into consideration important differences between them. For example, the drug model assumes a liner-dose response relationship and that the only source of the agent is in the trial, however, nutrients such as vitamin D do not follow these assumptions (Heaney, 2014). Furthermore, all clinical studies utilised a standard vitamin D dose rather than individual vitamin D dosing to research a desired final concentration, which is suggested to be more relevant than dose, per se, in regard to health outcomes (Grant and Boucher, 2017). Moreover, the principle method to determine an individual's vitamin D status currently is to measure serum levels of (25(OH)D), which may not be reflective on the active metabolite (1,25,(OH)2D), compounding limitations further (Holick, 2009). And finally, similar with the pre-clinical studies, the concoctions of oral vitamin D capsules also varied between groups which would likely impact on the bioavailability and rate of absorption

and therefore introduce a source of uncontrolled variation between studies (Grossmann and Tangpricha, 2010).

In conclusion, strong recommendations relating to the potential therapeutic benefit attributed to vitamin D in neurodegenerative disease cannot be made based on the evidence systematically reviewed and presented here. The available pre-clinical and observational literature indicated vitamin D may play a causal role in development of neurodegenerative disease. However, minimal evidence from clinical trials exists to suggest oral vitamin D supplementation improves disease outcomes in AD, PD and ALS. Moreover, the clinical evidence relating to MS indicates that therapeutic benefits from sun exposure could be attributed to independent UV factors, rather than solely vitamin D. If vitamin D supplementation is equipotent to UVR derived vitamin D, as research assumes, then vitamin D supplementation would be expected to improve disease outcome to the same extent. The evidence from our systematic review suggests any UVR driven beneficial effect in neurological disease, such as MS, cannot be attributed to vitamin D alone. To properly address this gap in the literature, further research should consider what other UVR mediated factors might be generated in the skin that could be signalling to the brain through the development of specialized sensors with advanced sensitivity and the ability to detect all signalling mediators induced by UVR. Research would also benefit from the development of techniques sufficient to detect and distinguish not only the different forms of vitamin D, but vitamin D in both an inactive and active state, in order to more accurately reflect actual bioavailable vitamin D levels. A key recommendation of this systematic review is to establish a consensus dose and dosing regimen for vitamin D supplementation; additionally, future studies should aim to quantify serum vitamin D and metabolites concentrations to ensure compliance and bioavailability. It is also important to properly

address the large number of confounding variables that may influence variation in vitamin D status, disease severity and any underlying interactions that may skew results. The use of high quality prospective or clinical trials is important to build the literature base for (or against) the use of vitamin D in AD, PD and ALS. It is also advised that future studies employ tests that go beyond the neurological deficit alone and include measures of other factors that may impact on vitamin D status, such as physical activity and diet.

Chapter 5. Discussion

5.1 Summary of thesis outcomes

The primary focus of this thesis has surrounded UV light as a biologically relevant initiator of cutaneous responses that have the capacity to affect the brain. UVR is an important environmental hazard across the globe, and greater understanding of the broader implications from UVR exposure are needed to fully elucidate and manage the potential for harm associated with overexposure/avoidance of UVR.

Chapter 2. A link between ultraviolet radiation and addiction

Certain individuals show the behavioural characteristics of addiction following frequent exposure to UVR. Additionally, pre-clinical studies have demonstrated that UVR exposure, in mice, leads to the activation of neurological pathways that are associated with addiction pathology. Together, providing compelling evidence that UVR can influence central neuronal circuits and affect behavioural outcomes in a manner that is comparable to addiction. However, clear understanding of the physiological mechanisms by which UVR can elicit downstream consequences is lacking and more research is needed to develop these concepts further. This includes exploring additional aspects of addiction pathology, such as the role of the immune system which is now recognised to increase the vulnerability to addiction. Thus, this thesis has investigated the converging lines of evidence surrounding cutaneous exposure to UVR, immune activation, and addiction, aiming to gain greater insights as to how UVR can access central tissue and influence neuronal circuitry. This has led to the development of a novel hypothesis that neuroimmune signalling, initiated by UV-damaged skin cells, contributes to potentiated signalling within the cortico-mesolimbic pathways and a subsequent addiction phenotype. This work has

been published in *Brain, Behaviour and Immunity* and raises the following questions; 1) is UVR driving a form of addiction and 2) is this mediated by peripherally induced alarmin signalling induced from damage sustained by UVR. These research questions were explored in Chapter 3.

Chapter 3. Does UV-induced alarmin signalling influence dopaminergic transmission and increase motivation?

In succession to the literature review undertaken in chapter 2, a research proposal detailing planned experiments to explore the role of the immune system in the reward-related behaviour following UV exposure is presented. The designed experimental works aims to examine whether UVR induced alarmin signalling, arising from UV-damaged skin cells, leads to increased reinforcement or motivation by effecting dopamine transmission within the NAc. The completion of this study will shed light on the role of the immune system and dopamine transmission in mediating the reinforcing effects of persistent UVR exposure. If a correlation between TLR4 activation and dopaminergic modification is found, this could have further ramifications for understanding how the immune system contributes to addictive and motivational behaviours.

Chapter 4. Does source of vitamin D influence neurological effects?

An important consideration to UV-related research is that UV-B wavelengths are responsible for the cutaneous production of vitamin D. As such, virtually all UVR exposure will result in increased vitamin D synthesis. Vitamin D exerts a number of biological effects throughout the body through interactions with the VDR. In particular, vitamin D has been suggested to protect against several neurological disorders. Thus, a systematic review was performed to explore if the neuroprotective benefits from vitamin D, in neurodegenerative disease, were dependent on the route of administration, by comparing published studies

that either supplemented with vitamin or provided a measure of sun-derived vitamin D. The findings indicated that there was insufficient data to comprehensively reflect on the neuroprotective potential for vitamin D and whether this is dependent on the route of administration. Solid clinical evidence supporting vitamin D as a neuroprotective agent is lacking, and the majority of current supportive data are based on preclinical and observational studies. Therefore, formal recommendations regarding therapeutic use of vitamin D, in neurodegenerative disease, cannot be made. The evidence presented in this systematic review also raises the question if vitamin D is the true mediator of the presumed therapeutic benefits associated with UVR exposure, or if other independent UVR factors are involved. To adequately address this gap in the literature, future research needs to consider alternative cutaneous responses to UVR, such as the alarmin response discussed in Chapter 2, and the potential for these mediators to also impact on neuronal tissues. The hypothesis and accompanying research proposal presented in Chapters 2 and 3 may shed light on potential independent UVR factors and their downstream effects.

5.2 General discussion and future directions

As UVR is biologically active, contributing to both positive and detrimental outcomes, UVR is relevant to health of individuals across the globe. The importance of UVR has been highlighted in recent years with factors such as migration and changes in lifestyle presenting with increased challengers associated with insufficient or over-exposure to UVR (Diffey, 2003; Jablonski and Chaplin, 2017). These factors also create the potential for new and diverse challenges to health and wellbeing that may be attributed to UVR but have previously been disregarded (Iacopetta et al., 2018). Furthermore, as the physiological responses to UVR are not limited to point of contact, but can reach distant sites, particularly the brain, the question of how UVR related signals travel throughout the

body is raised. Thus, the major theme throughout this thesis has surrounded the potential mechanisms by which peripheral, cutaneous UVR may influence central circuitry to modify behaviour and impact brain health.

The current body of work has suggested possible pathways by which UVR exposure may signal to the brain, affecting neurotransmission, through contact with the integumentary system. The skin receives continual UVR through daily exposure as it forms the body's outer protective layer, thus skin is the largest gateway for UVR penetrate the body and exert an effect (Autier et al., 2014; Becklund et al., 2010; Slominski, 2015). The interaction of UVR with skin cells contributes to an inflammatory state through UVR induced damage as well as causing endogenous production of vitamin D. Both inflammatory alarmins and vitamin D can travel throughout the body in the circulation, reaching distant sites, including the brain, rendering them prime candidates to facilitate UVR-mediated skin-to-brain communication. Throughout this thesis, clinically relevant examples have been established, demonstrating the potential for these overlapping pathways to modulate central tissue. Yet, exact molecular and cellular mechanisms by which cutaneous UVR results in neuronal modification remain elusive. More research is needed to define specific pathways and neural routes that are involved in translating peripheral-to-central signalling induced by UVR. The discovery of a clear molecular or cellular pathway by which UVR can lead to central modulation will advance the fields of photoimmunology and neuroscience. This may help to develop greater understanding of the systematic effects of UVR and broader implications for health and disease. The proposed experimental work presented in Chapter 3 is the first step in addressing if the immune system facilitates central alterations induced by UVR exposure. The completion of this study will provide clear insights on whether UVR influences dopaminergic systems

within the brain to increase motivated behaviour; and if this is driven, at least in part, by inflammatory signalling arising from UVR damaged skin cells.

To progress knowledge further and best investigate systemic UVR responses, whole systems that can be pharmacologically manipulated are needed in order to identify UVR induced changes that occur within living tissues. Providing the opportunity to observe peripheral responses to UVR, such as the photochemical reactions resulting in the release of alarmins and the production of vitamin D, and then track corresponding signalling pathways that result in central alterations. Thus, animal models are a critical tool to gain further insights to improve this understanding. Yet, it is important to identify that animal models are not without limitations. In the proposed study design presented in Chapter 3, Sprague Dawley rats were selected as they are cost effective, accessible and can be routinely trained to perform behavioural tasks. However, as Sprague Dawley rats are albino and nocturnal animals with limited melanin production, it is possible that UVR induced changes may not be directly translational to human responses and crucial components of the pathway could be unalike or missing completely. Hence, caution must be taken when interpreting results and additional studies may be needed to further validate identified mechanisms.

Traditionally, methods of administering UVR in animal models has been to suspend a UV emitting bulb which illuminates the entire cage, shining on the dorsal surface of shaved or hairless animals (Fell et al., 2014; Johnson et al., 2013; Skobowiat and Slominski, 2015). However, factors such as the distance of the light from the cage, the angle of light emissions, the presence of objects that may reflect or block light and differences in the

emission spectrum of individual light bulbs can lead to substantial variability in the amount of actual UVR received (Goettsch et al., 1999). For these reasons, making comparisons of the biological reactions induced by different UVR set ups is near impossible. In order to control variability and ensure we are reporting precise quantities of the UVR administered in our model, we have designed a new apparatus that guides UV light directly onto the skin via a dispersing cone, securely fitted to the animal (see Appendix A). The benefit of guiding the light guiding UVR directly onto the skin is that we can definitively say how much UVR the animal received within a given time period as no radiance is lost. Thus, this may provide a more accurate assessment of biological changes induced by different quantities of UVR.

Designing a secure-fitting apparatus was also paramount to minimise harm by protecting the most sensitive areas of the animal (eye, ears and paws) from exposure. As the light is guided directly onto a contained and target area of the skin, the animal is protected and potential confounding variables are eliminated. For example, existing models can be profoundly stressful if precautions to protect they eyes are not taken, due to the inescapable brightness unaccustomed to nocturnal animals (Bouwknecht et al., 2007; Nathiya and Vanisree, 2010). As much remains unknown about the role of the immune system in pathological conditions, it is necessary to control for variables such as stress which can also influence immune function (Liu et al., 2014).

A major challenge in all health-related UVR research has been to formulate a quantitative radiation-based dose-response relationship for UVR exposure and disease (Lucas and Ponsonby, 2002). Consequently, the optimal dose or amount of UVR exposure a person should receive remains unknown. This is partly due to epidemiological studies relying on recalled sun-exposure or actinic damage as measures of past exposure, which are highly subjective. It is also likely that for certain diseases, including melanoma and

basal cell carcinoma of the skin, there is not a simple linear equation between exposure and disease; factors such as the timing and pattern of exposure may critically influence disease outcomes (Armstrong and Kricker, 2001). Defining a relationship between UVR exposure and disease is further complicated as there is often a long lag period between risks factors and development of disease (Lucas et al., 2006). In addition, behavioural factors and skin pigmentation make it difficult to directly equate personal doses of UVR from physical measures of ambient UVR. Skin pigmentation alone can substantially alter the dose-response relationship, darker skin has a 33-fold higher minimal erythemal dose compared to fairer skin (Clydesdale et al., 2001). Whereas, behavioural factors such as clothing choices, the use of sun-screens and intentional sun-exposure can cause a 100-fold difference in personal UVR exposure (Gies et al., 2004).

Considerable advances need to occur in the vitamin D field to adequately address knowledge gaps and truly recognise the role of vitamin D in health and disease. Presently, there are a number of uncertainties surrounding the physiological function of vitamin D, the effects of long-term high/low doses of vitamin D, and the efficacy of different vitamin D metabolites. Moreover, vitamin D research is often limited due to the number of confounding variables associated with circulating vitamin D levels (e.g. physical activity, weight, age) and that definitions of vitamin D insufficiency/deficiency are not standardised (Taylor et al., 2017). The future of vitamin D research needs to improve detection methods in order to distinguish the different vitamin D metabolites (i.e. vitamin D2/D3) and better monitor active vitamin D levels. Future research would also benefit from designing studies that can separate vitamin D-dependent and -independent contributions from UVR.

5.3 Concluding remarks

In conclusion, UVR is an important modifiable risk factor for a range of diseases affecting human health. Despite the fact that UVR only comes in contact with two peripheral ports of entry, through the skin and eyes, it is evident that the effects from UVR extend systemically and can influence neuronal circuitry. Although, physiological mechanisms remain unclear, this thesis has explored two pathways by which the integumentary system may facilitate skin-to-brain signalling induced by UVR, and the implications of this for neurological disorders. The outcomes of this thesis have identified that in order to overcome the limitations associated with UVR and vitamin D-related research, more consistency is needed surrounding experimental design and controlling for the wide number of confounding variables associated with UVR exposure and with circulating levels of vitamin D. The work contained in this thesis has significant implications for progressing the understanding of how peripheral UVR, potentially via the immune system or the vitamin D pathway, can access central circuits and affect neuronal outcomes.

References

Abdolahinia, A., Sadr, M. & Hessami, Z. 2012. Correlation between the age of smoking initiation and maintaining continuous abstinence for 5 years after quitting. *Acta Med Iran,* 50**,** 755-759.

Achiron, A., Givon, U., Magalashvili, D., Dolev, M., Liraz Zaltzman, S., Kalron, A., Stern, Y., Mazor, Z., Ladkani, D. & Barak, Y. 2015. Effect of Alfacalcidol on multiple sclerosis-related fatigue: A randomized, double-blind placebo-controlled study. *Mult Scler,* 21**,** 767-775.

Adzemovic, M. Z., Zeitelhofer, M., Hochmeister, S., Gustafsson, S. A. & Jagodic, M. 2013. Efficacy of vitamin D in treating multiple sclerosis-like neuroinflammation depends on developmental stage. *Experimental Neurology,* 249**,** 39-48.

Afzal, S., Bojesen, S. E. & Nordestgaard, B. G. 2014. Reduced 25-hydroxyvitamin D and risk of Alzheimer's disease and vascular dementia. *Alzheimers Dementia,* 10**,** 296-302.

Akil, H., Owens, C., Gutstein, H., Taylor, L., Curran, E. & Watson, S. 1998. Endogenous opioids: overview and current issues. *Drug Alcohol Depend,* 51**,** 127-140.

Akira, S., Takeda, K. & Kaisho, T. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nature immunology,* 2**,** 675-680.

Alboni, S., Micioni Di Bonaventura, M. V., Benatti, C., Giusepponi, M. E., Brunello, N. & Cifani, C. 2017. Hypothalamic expression of inflammatory mediators in an animal model of binge eating. *Behav Brain Res,* 320**,** 420-430.

American Psychiatric Association 2013. *Diagnostic and statistical manual of mental disorders (DSM-5®),* Washinton, DC.

Anderson, F. 2005. Vitamin D for older people: how much, for whom and-above all-why? *Age and Ageing,* 34**,** 425-426.

Annweiler, C., Llewellyn, D. J. & Beauchet, O. 2013. Low serum vitamin D concentrations in Alzheimer's disease: A systematic review and meta-analysis. *Journal of Alzheimer's Disease,* 33**,** 659-674.

Annweiler, C., Rolland, Y., Schott, A., Blain, H., Vellas, B. & Beauchet, O. 2011. Serum vitamin D deficiency as a predictor of incident non-Alzheimer dementias: A 7-year longitudinal study. *Dementia and Geriatric Cognitive Disorders,* 32**,** 273-278.

Annweiler, C., Rolland, Y., Schott, A. M., Blain, H., Vellas, B., Herrmann, F. R. & Beauchet, O. 2012. Higher vitamin D dietary intake is associated with lower risk of Alzheimer's disease: A 7-year follow-up. *The Journals of Gerontology: Series A: Biological Sciences and Medical Sciences,* 67A**,** 1205-1211.

Antico, A., Tampoia, M., Tozzoli, R. & Bizzaro, N. 2012. Can supplementation with vitamin D reduce the risk or modify the course of autoimmune diseases? A systematic review of the literature. *Autoimmunity Reviews,* 12**,** 127-136.

Armas, L. A. G., Hollis, B. W. & Heaney, R. P. 2004. Vitamin D2 is much less effective than vitamin D3 in humans. *The Journal of Clinical Endocrinology & Metabolism,* 89**,** 5387-5391.

Armstrong, B. K. & Kricker, A. 2001. The epidemiology of UV induced skin cancer. *J Photochem Photobiol B,* 63**,** 8-18.

Arulsamy, A., Teng, J., Colton, H., Corrigan, F. & Collins-Praino, L. 2018. Evaluation of early chronic functional outcomes and their relationship to pre-frontal cortex and hippocampal pathology following moderate-severe traumatic brain injury. *Behav Brain Res,* 348**,** 127-138.

Aubert, P. M., Seibyl, J. P., Price, J. L. & Harris, T. S. 2016. Dopamine Efflux in Response to Ultraviolet Radiation in Addicted Sunbed Users. *Psychiatry Research: Neuroimaging,* 251**,** 7-14.

Autier, P., Boniol, M., Pizot, C. & Mullie, P. 2014. Vitamin D status and ill health: a systematic review. *Lancet Diabetes Endocrinol,* 2**,** 76-89.

Avena, N. M., Rada, P. & Hoebel, B. G. 2009. Sugar and fat bingeing have notable differences in addictive-like behavior. *The Journal of nutrition,* 139**,** 623-628.

Baarnhielm, M., Hedstrom, A., Kockum, I., Sundqvist, E., Gustafsson, S., Hillert, J., Olsson, T. & Alfredsson, L. 2012. Sunlight is associated with decreased multiple sclerosis risk: No interaction with human leukocyte antigen-DRB1*15. *European Journal of Neurology,* 19**,** 955-962.

Balfour, M. E., Yu, L. & Coolen, L. M. 2004. Sexual behavior and sex-associated environmental cues activate the mesolimbic system in male rats. *Neuropsychopharmacology,* 29**,** 718-730.

Banks, W. A., Ortiz, L., Plotkin, S. R. & Kastin, A. J. 1991. Human interleukin (IL) 1 alpha, murine IL-1 alpha and murine IL-1 beta are transported from blood to brain in the mouse by a shared saturable mechanism. *J Pharmacol Exp Ther,* 259**,** 988-996.

Bauman, A., Merom, D., Bull, F. C., Buchner, D. M. & Fiatarone Singh, M. A. 2016. Updating the Evidence for Physical Activity: Summative Reviews of the Epidemiological Evidence, Prevalence, and Interventions to Promote "Active Aging". *Gerontologist,* 56 Suppl 2**,** S268-280.

Becklund, B. R., Severson, K. S., Vang, S. V. & DeLuca, H. F. 2010. UV radiation suppresses experimental autoimmune encephalomyelitis independent of vitamin D production. *Proc Natl Acad Sci U S A,* 107**,** 6418-6423.

Beecher, M. E., Eggett, D., Erekson, D., Rees, L. B., Bingham, J., Klundt, J., Bailey, R. J., Ripplinger, C., Kirchhoefer, J., Gibson, R., Griner, D., Cox, J. C. & Boardman, R. D. 2016. Sunshine on my shoulders: Weather, pollution, and emotional distress. *J Affect Disord,* 205**,** 234-238.

Bello, N. T. & Hajnal, A. 2010. Dopamine and binge eating behaviors. *Pharmacology Biochemistry and Behavior,* 97**,** 25-33.

Bennett, C. L., Noordegraaf, M., Martina, C. A. & Clausen, B. E. 2007. Langerhans cells are required for efficient presentation of topically applied hapten to T cells. *J Immunol,* 179**,** 6830-6835.

Bennett, L., Kersaitis, C., Macaulay, S. L., Münch, G., Niedermayer, G., Nigro, J., Payne, M., Sheean, P., Vallotton, P., Zabaras, D. & Bird, M. 2013. Vitamin D2-Enriched Button Mushroom (Agaricus bisporus) Improves Memory in Both Wild Type and APPswe/PS1dE9 Transgenic Mice. *PLoS ONE,* 8.

Bergenmar, M. & Brandberg, Y. 2001. Sunbathing and sun-protection behaviors and attitudes of young Swedish adults with hereditary risk for malignant melanoma. *Cancer nursing,* 24**,** 341-350.

Bergh, C., Eklund, T., Södersten, P. & Nordin, C. 1997. Altered dopamine function in pathological gambling. *Psychological medicine,* 27**,** 473-475.

Berridge, K. C., Robinson, T. E. & Aldridge, J. W. 2009. Dissecting components of reward: 'liking', 'wanting', and learning. *Current opinion in pharmacology,* 9**,** 65-73.

Bianchi, M. E. 2007. DAMPs, PAMPs and alarmins: all we need to know about danger. *Journal of Leukocyte Biology,* 81**,** 1-5.

Bikle, D. 2000. Vitamin D: Production, Metabolism, and Mechanisms of Action. *In:* De Groot, L. J., Chrousos, G., Dungan, K., Feingold, K. R., Grossman, A., Hershman, J. M., Koch, C., Korbonits, M., McLachlan, R., New, M., Purnell, J., Rebar, R., Singer, F. & Vinik, A. (eds.) *Endotext.* South Dartmouth (MA): MDText.com, Inc.

Bikle, Daniel D. 2014. Vitamin D Metabolism, Mechanism of Action, and Clinical Applications. *Chemistry & Biology,* 21**,** 319-329.

Bilbo, S. D. & Schwarz, J. M. 2012. The immune system and developmental programming of brain and behavior. *Frontiers in neuroendocrinology,* 33**,** 267-286.

Bjornevik, K., Riise, T., Casetta, I., Drulovic, J., Granieri, E., Holmoy, T., Kampman, M. T., Landtblom, A. M., Lauer, K., Lossius, A., Magalhaes, S., Myhr, K. M., Pekmezovic, T., Wesnes, K., Wolfson, C. & Pugliatti, M. 2014. Sun exposure and multiple sclerosis risk in Norway and Italy: The EnvIMS study. *Mult Scler,* 20**,** 1042-1049.

Black, H. S. & Lambert, C. R. 2001. Radical reactions of carotenoids and potential influence on UV carcinogenesis. *Curr Probl Dermatol,* 29**,** 140-156.

Bluthe, R. M., Walter, V., Parnet, P., Laye, S., Lestage, J., Verrier, D., Poole, S., Stenning, B. E., Kelley, K. W. & Dantzer, R. 1994. Lipopolysaccharide induces sickness behaviour in rats by a vagal mediated mechanism. *C R Acad Sci III,* 317**,** 499-503.

Borowski, T., Kokkinidis, L., Merali, Z. & Anisman, H. 1998. Lipopolysaccharide, central in vivo biogenic amine variations, and anhedonia. *Neuroreport,* 9**,** 3797-3802.

Bouwknecht, J. A., Spiga, F., Staub, D. R., Hale, M. W., Shekhar, A. & Lowry, C. A. 2007. Differential effects of exposure to low-light or high-light open-field on anxiety-related behaviors: relationship to c-Fos expression in serotonergic and non-serotonergic neurons in the dorsal raphe nucleus. *Brain Res Bull,* 72**,** 32-43.

Brash, D. E., Rudolph, J. A., Simon, J. A., Lin, A., McKenna, G. J., Baden, H. P., Halperin, A. J. & Ponten, J. 1991. A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. *Proc Natl Acad Sci U S A,* 88**,** 10124-10128.

Breder, C. D., Dinarello, C. A. & Saper, C. B. 1988. Interleukin-1 immunoreactive innervation of the human hypothalamus. *Science,* 240**,** 321-324.

Brenner, M. & Hearing, V. J. 2008. The Protective Role of Melanin Against UV Damage in Human Skin. *Photochemistry and Photobiology,* 84**,** 539-549.

Bromberg-Martin, E. S., Matsumoto, M. & Hikosaka, O. 2010. Dopamine in motivational control: rewarding, aversive, and alerting. *Neuron,* 68**,** 815-834.

Budden, T. & Bowden, N. A. 2013. The role of altered nucleotide excision repair and UVB-induced DNA damage in melanomagenesis. *Int J Mol Sci,* 14**,** 1132-1151.

Byrne, S., Beaugie, C., O'Sullivan, C., Leighton, S. & Halliday, G. M. 2011. The immune-modulating cytokine and endogenous alarmin interleukin-33 is upregulated in skin exposed to inflammatory UVB radiation. *The American journal of pathology,* 179**,** 211-222.

Capuron, L. & Miller, A. H. 2011. Immune system to brain signaling: neuropsychopharmacological implications. *Pharmacology & therapeutics,* 130**,** 226-238.

Cass, W. A., Peters, L. E., Fletcher, A. M. & Yurek, D. M. 2014. Calcitriol promotes augmented dopamine release in the lesioned striatum of 6-hydroxydopamine treated rats. *Neurochem Res,* 39**,** 1467-1476.

Cayrol, C. & Girard, J. P. 2014. IL-33: an alarmin cytokine with crucial roles in innate immunity, inflammation and allergy. *Curr Opin Immunol,* 31**,** 31-37.

Chan, J. K., Roth, J., Oppenheim, J. J., Tracey, K. J., Vogl, T., Feldmann, M., Horwood, N. & Nanchahal, J. 2012. Alarmins: awaiting a clinical response. *The Journal of clinical investigation,* 122**,** 2711-2719.

Chen, S., Ma, Q., Krafft, P. R., Hu, Q., Rolland, W., 2nd, Sherchan, P., Zhang, J., Tang, J. & Zhang, J. H. 2013. P2X7R/cryopyrin inflammasome axis inhibition reduces neuroinflammation after SAH. *Neurobiol Dis,* 58**,** 296-307.

Ciriello, J. & Gutman, M. B. 1991. Functional identification of central pressor pathways originating in the subfornical organ. *Can J Physiol Pharmacol,* 69**,** 1035-1045.

Clydesdale, G. J., Dandie, G. W. & Muller, K. H. 2001. Ultraviolet light induced injury: Immunological and inflammatory effects. *Immunology and Cell Biology,* 79**,** 547-568.

Cock, P. J., Antao, T., Chang, J. T., Chapman, B. A., Cox, C. J., Dalke, A., Friedberg, I., Hamelryck, T., Kauff, F. & Wilczynski, B. 2009. Biopython: freely available Python tools for computational molecular biology and bioinformatics. *Bioinformatics,* 25**,** 1422-1423.

Cokkinides, V., Weinstock, M., Glanz, K., Albano, J., Ward, E. & Thun, M. 2006. Trends in sunburns, sun protection practices, and attitudes toward sun exposure protection and tanning among US adolescents, 1998-2004. *Pediatrics,* 118**,** 853-864.

Coller, J. K. & Hutchinson, M. R. 2012. Implications of central immune signaling caused by drugs of abuse: mechanisms, mediators and new therapeutic approaches for prediction and treatment of drug dependence. *Pharmacology & therapeutics,* 134**,** 219-245.

Collins-Praino, L. E., Podurgiel, S. J., Kovner, R., Randall, P. A. & Salamone, J. D. 2012. Extracellular GABA in globus pallidus increases during the induction of oral tremor by haloperidol but not by muscarinic receptor stimulation. *Behav Brain Res,* 234**,** 129-135.

Corcos, M., Guilbaud, O., Paterniti, S., Moussa, M., Chambry, J., Chaouat, G., Consoli, S. M. & Jeammet, P. 2003. Involvement of cytokines in eating disorders: a critical review of the human literature. *Psychoneuroendocrinology,* 28**,** 229-249.

Corwin, R. L., Avena, N. M. & Boggiano, M. M. 2011. Feeding and reward: Perspectives from three rat models of binge eating. *Physiology & Behavior,* 104**,** 87-97.

Crowe, F. L., Steur, M., Allen, N. E., Appleby, P. N., Travis, R. C. & Key, T. J. 2011. Plasma concentrations of 25-hydroxyvitamin D in meat eaters, fish eaters, vegetarians and vegans: results from the EPIC-Oxford study. *Public Health Nutr,* 14**,** 340-346.

Cui, R., Widlund, H. R., Feige, E., Lin, J. Y., Wilensky, D. L., Igras, V. E., D'Orazio, J., Fung, C. Y., Schanbacher, C. F., Granter, S. R. & Fisher, D. E. 2007. Central role of p53 in the suntan response and pathologic hyperpigmentation. *Cell,* 128**,** 853-864.

D'Hooghe M, B., Haentjens, P., Nagels, G., Garmyn, M. & De Keyser, J. 2012. Sunlight exposure and sun sensitivity associated with disability progression in multiple sclerosis. *Mult Scler,* 18**,** 451-459.

D'Mello, C., Riazi, K., Le, T., Stevens, K. M., Wang, A., McKay, D. M., Pittman, Q. J. & Swain, M. G. 2013. P-selectin-mediated monocyte-cerebral endothelium adhesive interactions link peripheral organ inflammation to sickness behaviors. *The Journal of neuroscience,* 33**,** 14878-14888.

D'Orazio, J., Jarrett, S., Amaro-Ortiz, A. & Scott, T. 2013. UV Radiation and the Skin. *International Journal of Molecular Sciences,* 14**,** 12222-12248.

Dalmay, F., Bhalla, D., Nicoletti, A., Cabrera-Gomez, J. A., Cabre, P., Ruiz, F., Druet-Cabanac, M., Dumas, M. & Preux, P. M. 2010. Multiple sclerosis and solar exposure before the age of 15 years: case-control study in Cuba, Martinique and Sicily. *Mult Scler,* 16**,** 899-908.

Dankers, W., Colin, E. M., van Hamburg, J. P. & Lubberts, E. 2016. Vitamin D in Autoimmunity: Molecular Mechanisms and Therapeutic Potential. *Front Immunol,* 7**,** 697.

Dantzer, R. 2001. Cytokine-Induced Sickness Behavior: Mechanisms and Implications. *Annals of the New York Academy of Sciences,* 933**,** 222-234.

Dantzer, R. 2004. Cytokine-induced sickness behaviour: a neuroimmune response to activation of innate immunity. *Eur J Pharmacol,* 500**,** 399-411.

Dantzer, R., BluthÉ, R. & Laye, S. 1998. Cytokines and sickness behavior. *Annals of the New York Academy of Sciences,* 840.

Dantzer, R., O'Connor, J. C., Freund, G. G., Johnson, R. W. & Kelley, K. W. 2008. From inflammation to sickness and depression: when the immune system subjugates the brain. *Nature reviews. Neuroscience,* 9**,** 46-56.

De Gruijl, F. 1999. Skin cancer and solar UV radiation. *European Journal of Cancer,* 35**,** 2003-2009.

De Gruijl, F. R., Van Kranen, H. J. & Mullenders, L. H. 2001. UV-induced DNA damage, repair, mutations and oncogenic pathways in skin cancer. *J Photochem Photobiol B,* 63**,** 19-27.

De Leo, J. A., Tawfik, V. L. & LaCroix-Fralish, M. L. 2006. The tetrapartite synapse: path to CNS sensitization and chronic pain. *Pain,* 122**,** 17-21.

Dean, E. D., Mexas, L. M., Capiro, N. L., McKeon, J. E., DeLong, M. R., Pennell, K. D., Doorn, J. A., Tangpricha, V., Miller, G. W. & Evatt, M. L. 2012. 25-

Hydroxyvitamin D depletion does not exacerbate MPTP-induced dopamine neuron damage in mice. *PLoS One,* 7**,** e39227.

Dennis, L. K., Lowe, J. B. & Snetselaar, L. G. 2009. Tanning behaviour among young frequent tanners is related to attitudes and not lack of knowledge about the dangers. *Health Education Journal,* 68**,** 232-243.

Derakhshandi, H., Etemadifar, M., Feizi, A., Abtahi, S. H., Minagar, A., Abtahi, M. A., Abtahi, Z. A., Dehghani, A., Sajjadi, S. & Tabrizi, N. 2013. Preventive effect of vitamin D3 supplementation on conversion of optic neuritis to clinically definite multiple sclerosis: a double blind, randomized, placebo-controlled pilot clinical trial. *Acta Neurol Belg,* 113**,** 257-263.

Di Chiara, G. & Imperato, A. 1988. Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proceedings of the National Academy of Sciences of the United States of America,* 85**,** 5274-5278.

Diffey, B. 2002. Sources and measurement of ultraviolet radiation. *Methods,* 28**,** 4-13.

Diffey, B. 2003. Climate change, ozone depletion and the impact on ultraviolet exposure of human skin. *Physics in Medicine and Biology,* 49**,** R1-11.

Diffey, B. L. & Kochevar, I. E. 2007. Basic prinicples of photobiology. *In:* Hönigsmann H., H. J. L., Lim H.W. (ed.) *Photodermatology.* USA, New York: Informa Healtcare

Dominiak, M., Swiecicki, L. & Rybakowski, J. 2015. Psychiatric hospitalizations for affective disorders in Warsaw, Poland: Effect of season and intensity of sunlight. *Psychiatry Res,* 229**,** 287-294.

Duan, S., Lv, Z., Fan, X., Wang, L., Han, F., Wang, H. & Bi, S. 2014. Vitamin D status and the risk of multiple sclerosis: a systematic review and meta-analysis. *Neurosci Lett,* 570**,** 108-113.

Dutia, R., Meece, K., Dighe, S., Kim, A. J. & Wardlaw, S. L. 2012. beta-Endorphin antagonizes the effects of alpha-MSH on food intake and body weight. *Endocrinology,* 153**,** 4246-4255.

Erridge, C. 2010. Endogenous ligands of TLR2 and TLR4: agonists or assistants? *J Leukoc Biol,* 87**,** 989-999.

Espinosa-Ramirez, G., Ordonez, G., Flores-Rivera, J. & Sotelo, J. 2014. Sunlight exposure and multiple sclerosis in a tropical country. *Neurol Res,* 36**,** 647-650.

Ewing, J. A. 1984. Detecting alcoholism. The CAGE questionnaire. *The journal of the American Medical Association,* 252**,** 1905-1907.

Eyo, U. B. & Wu, L. J. 2013. Bidirectional microglia-neuron communication in the healthy brain. *Neural plasticity,* 2013**,** 10.

Fabry, Z., Fitzsimmons, K. M., Herlein, J. A., Moninger, T. O., Dobbs, M. B. & Hart, M. N. 1993. Production of the cytokines interleukin 1 and 6 by murine brain microvessel endothelium and smooth muscle pericytes. *J Neuroimmunol,* 47**,** 23-34.

Farias, A. S., Spagnol, G. S., Bordeaux-Rego, P., Oliveira, C. O. F., Fontana, A. G. M., de Paula, R. F. O., Santos, M. P. A., Pradella, F., Moraes, A. S., Oliveira, E. C., Longhini, A. L. F., Rezende, A. C. S., Vaisberg, M. W. & Santos, L. M. B. 2013.

Vitamin D3 induces IDO+ tolerogenic DCs and enhances treg, reducing the severity of EAE. *CNS Neuroscience and Therapeutics,* 19**,** 269-277.

Farsani, Z. S., Behmanesh, M. & Sahraian, M. A. 2015. Interleukin-10 but not transforming growth factor-β1 gene expression is up-regulated by vitamin D treatment in multiple. *Journal of the Neurological Sciences,* 350**,** 18-23.

Feldman, S. R., Liguori, A., Rapp, S. R., Fleischer, A. B., Lang, W. & Kaur, m. 2004. Ultraviolet exposure is a reinforcing stimulus in frequenr indoor tanners. *Journal of American Academy of Dermatology,* 51**,** 45-51.

Feldmeyer, L., Keller, M., Niklaus, G., Hohl, D., Werner, S. & Beer, H.-D. 2007. The Inflammasome Mediates UVB-Induced Activation and Secretion of Interleukin-1β by Keratinocytes. *Current Biology,* 17**,** 1140-1145.

Fell, G. L., Robinson, K. C., Mao, J., Woolf, C. J. & Fisher, D. E. 2014. Skin β-endorphin mediates addiction to UV light. *Cell,* 157**,** 1527-1534.

Fernandes de Abreu, D. A., Eyles, D. & Féron, F. 2009. Vitamin D, a neuro-immunomodulator: Implications for neurodegenerative and autoimmune diseases. *Psychoneuroendocrinology,* 34**,** S265-S277.

Gangwar, A. K., Rawat, A., Tiwari, S., Tiwari, S. C., Narayan, J. & Tiwari, S. 2015. Role of Vitamin-D in the prevention and treatment of Alzheimer's disease. *Indian J Physiol Pharmacol,* 59**,** 94-99.

Garcion, E., Sindji, L., Montero-Menei, C., Andre, C., Brachet, P. & Darcy, F. 1998. Expression of inducible nitric oxide synthase during rat brain inflammation: regulation by 1,25-dihydroxyvitamin D3. *Glia,* 22**,** 282-294.

Garcion, E., Sindji, L., Nataf, S., Brachet, P., Darcy, F. & Montero-Menei, C. N. 2003. Treatment of experimental autoimmune encephalomyelitis in rat by 1,25-dihydroxyvitamin D3 lead to early effects within the central nervous system. *Acta Neuropathologica,* 105**,** 438-448.

Garcion, E., Wion-Barbot, N., Montero-Menei, C. N., Berger, F. & Wion, D. 2002. New clues about vitamin D functions in the nervous system. *Trends Endocrinol Metab,* 13**,** 100-105.

Garland, C. F., Garland, F. C., Gorham, E. D., Lipkin, M., Newmark, H., Mohr, S. B. & Holick, M. F. 2006. The role of vitamin D in cancer prevention. *Am J Public Health,* 96**,** 252-261.

Gelfand, J. M., Cree, B. A., McElroy, J., Oksenberg, J., Green, R., Mowry, E. M., Miller, J. W., Hauser, S. L. & Green, A. J. 2011. Vitamin D in African Americans with multiple sclerosis. *Neurology,* 76**,** 1824-1830.

Gianforcaro, A. & Hamadeh, M. J. 2012. Dietary vitamin D3 supplementation at 10x the adequate intake improves functional capacity in the G93A transgenic mouse model of ALS, a pilot study. *CNS Neurosci Ther,* 18**,** 547-557.

Gianforcaro, A., Solomon, J. A. & Hamadeh, M. J. 2013. Vitamin D(3) at 50x AI attenuates the decline in paw grip endurance, but not disease outcomes, in the G93A mouse model of ALS, and is toxic in females. *PLoS One,* 8**,** e30243.

Gies, P., Roy, C., Javorniczky, J., Henderson, S., Lemus-Deschamps, L. & Driscoll, C. 2004. Global Solar UV Index: Australian measurements, forecasts and comparison with the UK. *Photochemistry and Photobiology,* 79**,** 32-39.

Goettsch, W., Garssen, J., de Gruijl, F. R., Dortant, P. & van Loveren, H. 1999. Methods for exposure of laboratory animals to ultraviolet radiation. *Lab Anim,* 33**,** 58-67.

Golan, D., Halhal, B., Glass-Marmor, L., Staun-Ram, E., Rozenberg, O., Lavi, I., Dishon, S., Barak, M., Ish-Shalom, S. & Miller, A. 2013. Vitamin D supplementation for patients with multiple sclerosis treated with interferon-beta: a randomized controlled trial assessing the effect on flu-like symptoms and immunomodulatory properties. *BMC Neurol,* 13**,** 60.

Goodsell, D. S. 2001. The molecular perspective: ultraviolet light and pyrimidine dimers. *The Oncologist,* 6**,** 298-299.

Grace, P. M., Ramos, K. M., Rodgers, K. M., Wang, X., Hutchinson, M. R., Lewis, M. T., Morgan, K. N., Kroll, J. L., Taylor, F. R., Strand, K. A., Zhang, Y., Berkelhammer, D., Huey, M. G., Greene, L. I., Cochran, T. A., Yin, H., Barth, D. S., Johnson, K. W., Rice, K. C., Maier, S. F. & Watkins, L. R. 2014. Activation of adult rat CNS endothelial cells by opioid-induced toll-like receptor 4 (TLR4) signaling induces proinflammatory, biochemical, morphological, and behavioral sequelae. *Neuroscience,* 280**,** 299-317.

Graeber, M. B. 2010. Changing face of microglia. *Science* 330**,** 783-788.

Grammatopoulos, D. K. & Chrousos, G. P. 2002. Functional characteristics of CRH receptors and potential clinical applications of CRH-receptor antagonists. *Trends Endocrinol Metab,* 13**,** 436-444.

Grange, F., Mortier, L., Crine, A., Robert, C., Sassolas, B., Lebbe, C., Lhomel, C. & Saiag, P. 2015. Prevalence of sunbed use, and characteristics and knowledge of sunbed users: results from the French population-based Edifice Melanoma survey. *Journal of the European Academy of Dermatology and Venereology* 29 Suppl 2**,** 23-30.

Grant, W. B. 2016. The role of geographical ecological studies in identifying diseases linked to UVB exposure and/or vitamin D. *Dermatoendocrinol,* 8**,** e1137400-1137423.

Grant, W. B. & Boucher, B. J. 2017. Randomized controlled trials of vitamin D and cancer incidence: A modeling study. *PLoS One,* 12**,** e0176448.

Gray, C., Gibbons, R., Larouche, R., Sandseter, E. B., Bienenstock, A., Brussoni, M., Chabot, G., Herrington, S., Janssen, I., Pickett, W., Power, M., Stanger, N., Sampson, M. & Tremblay, M. S. 2015. What Is the Relationship between Outdoor Time and Physical Activity, Sedentary Behaviour, and Physical Fitness in Children? A Systematic Review. *Int J Environ Res Public Health,* 12**,** 6455-6474.

Grimbaldeston, M. A., Nakae, S., Kalesnikoff, J., Tsai, M. & Galli, S. J. 2007. Mast cell–derived interleukin 10 limits skin pathology in contact dermatitis and chronic irradiation with ultraviolet B. *Nature Immunology,* 8**,** 1095-1104.

Grimm, M. O. W., Thiel, A., Lauer, A. A., Winkler, J., Lehmann, J., Regner, L., Nelke, C., Janitschke, D., Benoist, C., Streidenberger, O., Stotzel, H., Endres, K., Herr, C., Beisswenger, C., Grimm, H. S., Bals, R., Lammert, F. & Hartmann, T. 2017. Vitamin D and Its Analogues Decrease Amyloid-beta (Abeta) Formation and Increase Abeta-Degradation. *Int J Mol Sci,* 18.

Grossmann, R. E. & Tangpricha, V. 2010. Evaluation of vehicle substances on vitamin D bioavailability: a systematic review. *Molecular nutrition & food research,* 54**,** 1055-1061.

Guttmannova, K., Bailey, J. A., Hill, K. G., Lee, J. O., Hawkins, J. D., Woods, M. L. & Catalano, R. F. 2011. Sensitive periods for adolescent alcohol use initiation: predicting the lifetime occurrence and chronicity of alcohol problems in adulthood. *J Stud Alcohol Drugs,* 72**,** 221-231.

Guy, G. P., Berkowitz, Z., Everett Jones, S., Holman, D. M., Garnett, E. & Watson, M. 2015. Trends in indoor tanning among US high school students, 2009-2013. *The journal of the American Medical Association,* 151**,** 448-450.

Harrington, C. R., Beswick, T. C., Graves, M., Jacob, H. T., Harris, T. S., Kourash, S., Devous, M. D. & Adinoff, B. 2011a. Activation of the mesostriatal reward pathway with exposure to ultraviolet radiation (UVR) vs. sham UVR in frequent tanners: a pilot study. *Addiction Biology,* 17**,** 680-686.

Harrington, C. R., Beswick, T. C., Leitenberger, J., Minhajuddin, A., Jacobe, H. T. & Adinoff, B. 2011b. Addictive-like behaviours to ultraviolet light among frequent indoor tanners. *Clinical and Experimental Dermatology,* 36**,** 33-38.

Hart, P. H., Gorman, S. & Finlay-Jones, J. J. 2011. Modulation of the immune system by UV radiation: more than just the effects of vitamin D? *Nat Rev Immunol,* 11**,** 584-596.

Hayward, M. G. & Keatinge, W. R. 1981. Roles of subcutaneous fat and thermoregulatory reflexes in determining ability to stabilize body temperature in water. *J Physiol,* 320**,** 229-251.

Heaney, R. P. 2014. Guidelines for optimizing design and analysis of clinical studies of nutrient effects. *Nutr Rev,* 72**,** 48-54.

Heber, D. & Carpenter, C. L. 2011. Addictive Genes and the Relationship to Obesity and Inflammation. *Molecular Neurobiology,* 44**,** 160.

Heckman, C. J., Chandler, R., Kloss, J. D., Benson, A., Rooney, D., Munshi, T., Darlow, S. D., Perlis, C., Manne, S. L. & Oslin, D. W. 2013. Minimal Erythema Dose (MED) testing. *J Vis Exp***,** e50175.

Hejazi, E., Amani, R., Sharafodinzadeh, N. & Cheraghian, B. 2014. Comparison of antioxidant status and vitamin D levels between multiple sclerosis patients and healthy matched subjects. *Multiple Sclerosis International,* 2014**,** 539854.

Hill, M. A. 2018. Embryology *In:* 01, A. e. h. (ed.) *https://embryology.med.unsw.edu.au/embryology/index.php/File:Adult_epidermis_histology_01.jpg.*

Hillhouse, J. J., Baker, M., Turrisi, R., Shields, A., Stapleton, J., Jain, S. & Longacre, I. 2012. Evaluating a Measure of Tanning Abuse and Dependence. *Archives of Dermatology,* 148**,** 815-819.

Hines, D. J., Choi, H. B., Hines, R. M., Phillips, A. G. & MacVicar, B. A. 2013. Prevention of LPS-induced microglia activation, cytokine production and sickness behavior with TLR4 receptor interfering peptides. *PloS one,* 8**,** 10.

Hoel, D. G., Berwick, M., de Gruijl, F. R. & Holick, M. F. 2016. The risks and benefits of sun exposure 2016. *Dermatoendocrinol,* 8**,** 17.

Holden, R. J. & Pakula, I. S. 1996. The role of tumor necrosis factor-alpha in the pathogenesis of anorexia and bulimia nervosa, cancer cachexia and obesity. *Med Hypotheses,* 47**,** 423-438.

Holick, M. F. 2009. Vitamin D status: measurement, interpretation, and clinical application. *Ann Epidemiol,* 19**,** 73-78.

Holick, M. F. 2011. Vitamin D: A D-lightful solution for health. *Journal of Investigative Medicine,* 59**,** 872-880.

Holick, M. F. 2016. Biological Effects of Sunlight, Ultraviolet Radiation, Visible Light, Infrared Radiation and Vitamin D for Health. *Anticncer research* 36**,** 1345-1356.

Holick, M. F., MacLaughlin, J. A. & Doppelt, S. H. 1981. Regulation of cutaneous previtamin D3 photosynthesis in man: skin pigment is not an essential regulator. *Science,* 211**,** 590-593.

Holliman, G., Lowe, D., Cohen, H., Felton, S. & Raj, K. 2017. Ultraviolet Radiation-Induced Production of Nitric Oxide:A multi-cell and multi-donor analysis. *Sci Rep,* 7**,** 11105.

Hönigsmann, H. 2002. Erythema and pigmentation. *Photodermatology, photoimmunology & photomedicine,* 18**,** 75-81.

Horst, R. L., Reinhardt, T. A. & Ramberg, C. F. 1986. 24-Hydroxylation of 1, 25-dihydroxyergocalciferol. An unambiguous deactivation process. *The Journal of biological chemistry,* 261**,** 9250-9256.

Hossein-nezhad, A., Spira, A. & Holick, M. F. 2013. Influence of vitamin D status and vitamin D3 supplementation on genome wide expression of white blood cells: a randomized double-blind clinical trial. *PLoS One,* 8.

Houghton, L. A. & Vieth, R. 2006. The case against ergocalciferol (vitamin D2) as a vitamin supplement. *Am J Clin Nutr,* 84**,** 694-697.

Hunt, Y., Augustson, E., Rutten, L. & Moser, R. 2012. History and culture of tanning in the United States. *In:* Heckman, C. J. & Manne, S. L. (eds.) *Shedding Light on Indoor Tanning.* Dordrecht: Springer Netherlands.

Hutchinson, M. R., Northcutt, A. L. & Chao, L. W. 2008. Minocycline suppresses morphine-induced respiratory depression, suppresses morphine-induced reward, and enhances systemic morphine-induced analgesia. *Brain,* 22**,** 1248-1256.

Hutchinson, M. R., Northcutt, A. L., Hiranita, T., Wang, X., Lewis, S. S., Thomas, J., van Steeg, K., Kopajtic, T. A., Loram, L. C., Sfregola, C., Galer, E., Miles, N. E., Bland, S. T., Amat, J., Rozeske, R. R., Maslanik, T., Chapman, T. R., Strand, K. A., Fleshner, M., Bachtell, R. K., Somogyi, A. A., Yin, H., Katz, J. L., Rice, K. C., Maier, S. F. & Watkins, L. R. 2012. Opioid Activation of Toll-Like Receptor 4 Contributes to Drug Reinforcement. *The Journal of Neuroscience,* 32**,** 11187-11200.

Hutchinson, M. R. & Watkins, L. R. 2014. Why is neuroimmunopharmacology crucial for the future of addiction research? *Neuropharmacology,* 76**,** 218-227.

Iacopetta, K., Collins-Praino, L. E., Buisman-Pijlman, F. T. A. & Hutchinson, M. R. 2018. Can neuroimmune mechanisms explain the link between ultraviolet light (UV) exposure and addictive behavior? *Brain Behav Immun***,** 125-132.

Ichihashi, M., Ueda, M., Budiyanto, A., Bito, T. & Oka, M. 2003. UV-induced skin damage. *Toxicology,* 199**,** 21-39.

Inoue, K., Hosoi, J. & Denda, M. 2007. Extracellular ATP has stimulatory effects on the expression and release of IL-6 via purinergic receptors in normal human epidermal keratinocytes. *J Invest Dermatol,* 127**,** 362-371.

Institute of Medicine Standing Committee on the Scientific Evaluation of Dietary Reference, I. 1997. The National Academies Collection: Reports funded by National Institutes of Health. *Dietary Reference Intakes for Calcium, Phosphorus, Magnesium, Vitamin D, and Fluoride.* Washington (DC): National Academies Press (US) National Academy of Sciences.

Ishiwari, K., Mingote, S., Correa, M., Trevitt, J. T., Carlson, B. B. & Salamone, J. D. 2004. The GABA uptake inhibitor beta-alanine reduces pilocarpine-induced tremor and increases extracellular GABA in substantia nigra pars reticulata as measured by microdialysis. *J Neurosci Methods,* 140**,** 39-46.

Islam, T., Gauderman, W. J., Cozen, W. & Mack, T. M. 2007. Childhood sun exposure influences risk of multiple sclerosis in monozygotic twins. *Neurology,* 69**,** 381-388.

Jablonski, N. G. & Chaplin, G. 2010. Colloquium paper: human skin pigmentation as an adaptation to UV radiation. *Proc Natl Acad Sci U S A,* 107 Suppl 2**,** 8962-8968.

Jablonski, N. G. & Chaplin, G. 2017. The colours of humanity: the evolution of pigmentation in the human lineage. *Philos Trans R Soc Lond B Biol Sci,* 372.

Jacobsen, J. H., Watkins, L. R. & Hutchinson, M. R. 2014. Discovery of a novel site of opioid action at the innate immune pattern-recognition receptor TLR4 and its role in addiction. *International review of neurobiology,* 118**,** 129-163.

Jans, J., Garinis, G. A., Schul, W., van Oudenaren, A., Moorhouse, M., Smid, M., Sert, Y.-G., van der Velde, A., Rijksen, Y., de Gruijl, F. R., van der Spek, P. J., Yasui, A., Hoeijmakers, J. H. J., Leenen, P. J. M. & van der Horst, G. T. J. 2006. Differential Role of Basal Keratinocytes in UV-Induced Immunosuppression and Skin Cancer. *Molecular and Cellular Biology,* 26**,** 8515-8526.

Japelt, R. B. & Jakobsen, J. 2013. Vitamin D in plants: a review of occurrence, analysis, and biosynthesis. *Front Plant Sci,* 4**,** 20.

Jelinek, G. A., Marck, C. H., Weiland, T. J., Pereira, N., van der Meer, D. M. & Hadgkiss, E. J. 2015. Latitude, sun exposure and vitamin D supplementation: Associations with quality of life and disease outcomes in a large international cohort of people with multiple sclerosis. *BMC Neurology* 15**,** 6.

Jianping, C., Paredes, W. & Lowinson, J. H. 1990. Δ9-Tetrahydroeannabinol enhances presynaptic dopamine efflux in medial prefrontal cortex. *European journal of Pharmacology,* 190**,** 259-262.

Johnson, K. E., Wulff, B. C., Oberyszyn, T. M. & Wilgus, T. A. 2013. Ultraviolet light exposure stimulates HMGB1 release by keratinocytes. *Archives of dermatological research,* 305**,** 805-815.

Jonak, C., Klosner, G. & Trautinger, F. 2009. Significance of heat shock proteins in the skin upon UV exposure. *Front Biosci (Landmark Ed),* 14**,** 4758-4768.

Juzeniene, A., Brekke, P., Dahlback, A., Andersson-Engels, S., Reichrath, J., Moan, K., Holick, M. F., Grant, W. B. & Moan, J. 2011. Solar radiation and human health. *Reports on Progress in Physics,* 74**,** 56.

Kampman, M. T., Steffensen, L. H., Mellgren, S. I. & Jorgensen, L. 2012. Effect of vitamin D3 supplementation on relapses, disease progression, and measures of function in persons with multiple sclerosis: exploratory outcomes from a double-blind randomised controlled trial. *Mult Scler,* 18**,** 1144-1151.

Kampman, M. T., Wilsgaard, T. & Mellgren, S. I. 2007. Outdoor activities and diet in childhood and adolescence relate to MS risk above the Arctic Circle. *J Neurol,* 254**,** 471-477.

Karam, C., Barrett, M. J., Imperato, T., MacGowan, D. J. & Scelsa, S. 2013. Vitamin D deficiency and its supplementation in patients with amyotrophic lateral sclerosis. *J Clin Neurosci,* 20**,** 1550-1553.

Kaur, M., Liguori, A., Fleischer, A. B. & Feldman, S. R. 2005. Side effects of naltrexone observed in frequent tanners: could frequent tanners have ultraviolet-induced high opioid levels? *Journal of the American Academy of Dermatology,* 52**,** 916.

Kaur, M., Liguori, A., Fleischer, A. B. & Feldman, S. R. 2006. Plasma beta-endorphin levels in frequent and infrequent tanners before and after ultraviolet and non-ultraviolet stimuli. *Journal of the American Academy of Dermatology,* 54**,** 919-920.

Keller, M. C., Fredrickson, B. L., Ybarra, O., Cote, S., Johnson, K., Mikels, J., Conway, A. & Wager, T. 2005. A warm heart and a clear head. The contingent effects of weather on mood and cognition. *Psychol Sci,* 16**,** 724-731.

Kelley, K. W., Bluthé, R.-M., Dantzer, R., Zhou, J.-H., Shen, W.-H., Johnson, R. W. & Broussard, S. R. 2003. Cytokine-induced sickness behavior. *Brain, behavior, and immunity,* 17**,** S112-S118.

Keyse, S. M. & Tyrrell, R. M. 1989. Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. *Proc Natl Acad Sci U S A,* 86**,** 99-103.

Khuder, S. A., Dayal, H. H. & Mutgi, A. B. 1999. Age at smoking onset and its effect on smoking cessation. *Addict Behav,* 24**,** 673-677.

Kift, R., Berry, J. L., Vail, A., Durkin, M. T., Rhodes, L. E. & Webb, A. R. 2013. Lifestyle factors including less cutaneous sun exposure contribute to starkly lower vitamin D levels in U.K. South Asians compared with the white population. *Br J Dermatol,* 169**,** 1272-1278.

Kim, J. S., Ryu, S. Y., Yun, I., Kim, W. J., Lee, K. S., Park, J. W. & Kim, Y. I. 2006. 1alpha,25-Dihydroxyvitamin D(3) Protects Dopaminergic Neurons in Rodent Models of Parkinson's Disease through Inhibition of Microglial Activation. *J Clin Neurol,* 2**,** 252-257.

Knight, J. M., Kirincich, A. N., Farmer, E. R. & Hood, A. F. 2002. Awareness of the risks of tanning lamps does not influence behavior among college students. *Archives of dermatology,* 138**,** 1311-1315.

Knippenberg, S., Damoiseaux, J., Bol, Y., Hupperts, R., Taylor, B. V., Ponsonby, A. L., Dwyer, T., Simpson, S. & van der Mei, I. A. 2014. Higher levels of reported sun exposure, and not vitamin D status, are associated with less depressive symptoms and fatigue in multiple sclerosis. *Acta Neurol Scand,* 129**,** 123-131.

Koch, M., Schmid, A. & Schnitzler, H. U. 2000. Role of muscles accumbens dopamine D1 and D2 receptors in instrumental and Pavlovian paradigms of conditioned reward. *Psychopharmacology (Berl),* 152**,** 67-73.

Koch, S., Kohl, K., Klein, E., von Bubnoff, D. & Bieber, T. 2006. Skin homing of Langerhans cell precursors: adhesion, chemotaxis, and migration. *J Allergy Clin Immunol,* 117**,** 163-168.

Koduah, P., Paul, F. & Dorr, J. M. 2017. Vitamin D in the prevention, prediction and treatment of neurodegenerative and neuroinflammatory diseases. *Epma j,* 8**,** 313-325.

Komaki, G., Arimura, A. & Koves, K. 1992. Effect of intravenous injection of IL-1 beta on PGE2 levels in several brain areas as determined by microdialysis. *Am J Physiol,* 262**,** E246-251.

Kourosh, A. S., Harrington, C. R. & Adinoff, B. 2010. Tanning as a behavioral addiction. *The American journal of drug and alcohol abuse,* 36**,** 284-290.

Kragt, J. J., van Amerongen, B. M., Killestein, J., Dijkstra, C. D., Uitdehaag, B. M. J., Polman, C. H. & Lips, P. 2009. Higher levels of 25-hydroxyvitamin D are associated with a lower incidence of multiple sclerosis only in women. *Multiple Sclerosis,* 15**,** 9-15.

Kwon, E., Gallagher, L. G., Searles Nielsen, S., Franklin, G. M., Littell, C. T., Longstreth, W. T., Jr., Swanson, P. D. & Checkoway, H. 2013. Parkinson's disease and history of outdoor occupation. *Parkinsonism Relat Disord,* 19**,** 1164-1166.

Laethem, V. A., Claerhout, S. & Garmyn, M. 2005. The sunburn cell: regulation of death and survival of the keratinocyte. *The International Journal of Biochemistry & Cell Biology* 37**,** 1547-1553.

Laflamme, N. & Rivest, S. 1999. Effects of systemic immunogenic insults and circulating proinflammatory cytokines on the transcription of the inhibitory factor κBα within specific cellular. *Journal of neurochemistry,* 73**,** 309-321.

Landel, V., Millet, P., Baranger, K., Loriod, B. & Feron, F. 2016. Vitamin D interacts with Esr1 and Igf1 to regulate molecular pathways relevant to Alzheimer's disease. *Mol Neurodegener,* 11**,** 22.

Laube, J. C., Newland, M. J., Hogan, C., Brenninkmeijer, C. A. M., Fraser, P. J., Martinerie, P., Oram, D. E., Reeves, C. E., Röckmann, T., Schwander, J., Witrant, E. & Sturges, W. T. 2014. Newly detected ozone-depleting substances in the atmosphere. *Nature Geoscience,* 7**,** 266.

Leary, M. R. & Jones, J. L. 1993. The Social Psychology of Tanning and Sunscreen Use: Self-Presentational Motives as a Predictor of Health Risk1. *Journal of Applied Social Psychology,* 23**,** 1390-1406.

Lee, C. H., Wu, S. B., Hong, C. H., Yu, H. S. & Wei, Y. H. 2013. Molecular Mechanisms of UV-Induced Apoptosis and Its Effects on Skin Residential Cells: The Implication in UV-Based Phototherapy. *Int J Mol Sci,* 14**,** 6414-6435.

Leighton, S., Kok, L. F., Halliday, G. M. & Byrne, S. N. 2013. Inhibition of UV-induced uric acid production using allopurinol prevents suppression of the contact hypersensitivity response. *Exp Dermatol,* 22**,** 189-194.

Lin, J. Y. & Fisher, D. E. 2007. Melanocyte biology and skin pigmentation. *Melanocyte biology and skin pigmentation,* 445**,** 843-850.

Liu, J., Buisman-Pijlman, F. & Hutchinson, M. R. 2014. Toll-like receptor 4: innate immune regulator of neuroimmune and neuroendocrine interactions in stress and major depressive disorder. *Frontiers in neuroscience,* 8**,** 15.

Livingston, P. M., White, V., Hayman, J. & Dobbinson, S. 2003. Sun exposure and sun protection behaviours among Australian adolescents: trends over time. *Preventive Medicine,* 37**,** 577-584.

Loken-Amsrud, K. I., Holmoy, T., Bakke, S. J., Beiske, A. G., Bjerve, K. S., Bjornara, B. T., Hovdal, H., Lilleas, F., Midgard, R., Pedersen, T., Benth, J. S., Sandvik, L., Torkildsen, O., Wergeland, S. & Myhr, K.-M. 2012. Vitamin D and disease activity in multiple sclerosis before and during interferon-beta treatment. *Neurology,* 79**,** 267-273.

Lucas, R., McMichael, T., Smith, W. & Armstrong, B. K. 2006. Solar ultraviolet radiation: Global burden of disease from solar ultraviolet radiation. *In:* Prüss-Üstün, A., Zeeb, H., Mathers, C. & Repacholi, M. (eds.) *Environmental Burden of Disease Series, No.13.* Geneva: World Health Organisation.

Lucas, R. M. & Ponsonby, A. L. 2002. Ultraviolet radiation and health: friend and foe. *Med J Aust,* 177**,** 594-598.

Lucas, R. M., Ponsonby, A. L., Dear, K., Valery, P. C., Pender, M. P., Taylor, B. V., Kilpatrick, T. J., Dwyer, T., Coulthard, A., Chapman, C., van der Mei, I., Williams, D. & McMichael, A. J. 2011. Sun exposure and vitamin D are independent risk factors for CNS demyelination. *Neurology,* 76**,** 540-548.

Lugo, L. M., Lei, P. & Andreadis, S. T. 2011. Vascularization of the dermal support enhances wound re-epithelialization by in situ delivery of epidermal keratinocytes. *Tissue Eng Part A,* 17**,** 665-675.

Lupien, S. J., McEwen, B. S., Gunnar, M. R. & Heim, C. 2009. Effects of stress throughout the lifespan on the brain, behaviour and cognition. *Nat Rev Neurosci,* 10**,** 434-445.

Maier, S. F. 2003. Bi-directional immune-brain communication: Implications for understanding stress, pain, and cognition. *Brain Behav Immun,* 17**,** 69-85.

Mandia, D., Ferraro, O. E., Nosari, G., Montomoli, C., Zardini, E. & Bergamaschi, R. 2014. Environmental factors and multiple sclerosis severity: a descriptive study. *Int J Environ Res Public Health,* 11**,** 6417-6432.

Marrot, L. & Meunier, J. R. 2008. Skin DNA photodamage and its biological consequences. *J Am Acad Dermatol,* 58**,** S139-148.

Martinon, F., Pétrilli, V., Mayor, A., Tardivel, A. & Nature, T.-J. 2006. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature,* 440**,** 237-241.

Marx, S. J., Jones, G. & Weinstein, R. S. 1989. Differences in mineral metabolism among nonhuman primates receiving diets with only vitamin D3 or only vitamin D2. *The Journal of Clinical Endocrinology & Metabolism,* 69**,** 1282-1290.

Masoumi, A., Goldenson, B., Ghirmai, S., Avagyan, H., Zaghi, J., Abel, K., Zheng, X., Espinosa-Jeffrey, A., Mahanian, M., Liu, P. T., Hewison, M., Mizwickie, M., Cashman, J. & Fiala, M. 2009. 1alpha,25-dihydroxyvitamin D3 interacts with

curcuminoids to stimulate amyloid-beta clearance by macrophages of Alzheimer's disease patients. *J Alzheimers Dis,* 17**,** 703-717.

Matsumura, Y. 2002. Short-term and long-term cellular and molecular events following UV irradiation of skin: implications for molecular medicine. *Expert Reviews in Molecular Medicine,* 4**,** 1-22.

McCusker, R. H. & Kelley, K. W. 2013. Immune-neural connections: how the immune system's response to infectious agents influences behavior. *The Journal of experimental biology,* 216**,** 84-98.

McDowell, T. Y., Amr, S., Culpepper, W. J., Langenberg, P., Royal, W., Bever, C. & Bradham, D. D. 2011a. Sun exposure, vitamin D and age at disease onset in relapsing multiple sclerosis. *Neuroepidemiology,* 36**,** 39-45.

McDowell, T. Y., Amr, S., Culpepper, W. J., Langenberg, P., Royal, W., Bever, C. & Bradham, D. D. 2011b. Sun exposure, vitamin D intake and progression to disability among veterans with progressive multiple sclerosis. *Neuroepidemiology,* 37**,** 52-57.

McInnes, M. D. F., Moher, D., Thombs, B. D., McGrath, T. A., Bossuyt, P. M., Clifford, T., Cohen, J. F., Deeks, J. J., Gatsonis, C., Hooft, L., Hunt, H. A., Hyde, C. J., Korevaar, D. A., Leeflang, M. M. G., Macaskill, P., Reitsma, J. B., Rodin, R., Rutjes, A. W. S., Salameh, J. P., Stevens, A., Takwoingi, Y., Tonelli, M., Weeks, L., Whiting, P. & Willis, B. H. 2018. Preferred Reporting Items for a Systematic Review and Meta-analysis of Diagnostic Test Accuracy Studies: The PRISMA-DTA Statement. *Jama,* 319**,** 388-396.

McKinley, M. J., Hards, D. K. & Oldfield, B. J. 1994. Identification of neural pathways activated in dehydrated rats by means of Fos-immunohistochemistry and neural tracing. *Brain Res,* 653**,** 305-314.

Mead, M. N. 2008. Benefits of sunlight: a bright spot for human health. *Environ Health Perspect,* 116**,** A160-167.

Melendez, R. I. & Rodd-Henricks, Z. A. 2002. Microdialysis of dopamine in the nucleus accumbens of alcohol-preferring (P) rats during anticipation and operant self-administration of ethanol. *Alcoholism: Clinical and Experimental Research* 26**,** 318-325.

Miller, L. S. 2008. Toll-like receptors in skin. *Adv Dermatol,* 24**,** 71-87.

Miller, W. L. & Auchus, R. J. 2011. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocr Rev,* 32**,** 81-151.

Miller, Y. I., Choi, S. H., Wiesner, P., Fang, L., Harkewicz, R., Hartvigsen, K., Boullier, A., Gonen, A., Diehl, C. J., Que, X., Montano, E., Shaw, P. X., Tsimikas, S., Binder, C. J. & Witztum, J. L. 2011. Oxidation-specific epitopes are danger-associated molecular patterns recognized by pattern recognition receptors of innate immunity. *Circ Res,* 108**,** 235-248.

Milligan, E. D. & Watkins, L. R. 2009. Pathological and protective roles of glia in chronic pain. *Nature reviews. Neuroscience,* 10**,** 23-36.

Miyake, Y., Tanaka, K., Fukushima, W., Sasaki, S., Kiyohara, C., Tsuboi, Y., Yamada, T., Oeda, T., Miki, T., Kawamura, N., Sakae, N., Fukuyama, H., Hirota, Y., Nagai, M. & Fukuoka Kinki Parkinson's Disease Study, G. 2011. Lack of association of dairy

food, calcium, and vitamin D intake with the risk of Parkinson's disease: a case-control study in Japan. *Parkinsonism Relat Disord,* 17**,** 112-116.

Moghimi, E., Solomon, J. A., Gianforcaro, A. & Hamadeh, M. J. 2015. Dietary Vitamin D3 Restriction Exacerbates Disease Pathophysiology in the Spinal Cord of the G93A Mouse Model of Amyotrophic Lateral Sclerosis. *PLoS One,* 10**,** 23.

Montague, M., Borland, R. & Sinclair, C. 2001. Slip! Slop! Slap! and SunSmart, 1980-2000: Skin Cancer Control and 20 Years of Population-Based Campaigning. *Health Education & Behavior,* 28**,** 290-305.

Montecino, M., Stein, G. S., Stein, J. L., Lian, J. B., van Wijnen, A. J., Carvallo, L., Marcellini, S., Cruzat, F. & Arriagada, G. 2008. Vitamin D control of gene expression: temporal and spatial parameters for organization of the regulatory machinery. *Crit Rev Eukaryot Gene Expr,* 18**,** 163-172.

Mosayebi, G., Ghazavi, A., Ghasami, K., Jand, Y. & Kokhaei, P. 2011. Therapeutic effect of vitamin D3 in multiple sclerosis patients. *Immunol Invest,* 40**,** 627-639.

Mpandzou, G., Ben Haddou, E., Regragui, W., Benomar, A. & Yahyaoui, M. 2016. Vitamin D deficiency and its role in neurological conditions: A review. *Revue Neurologique,* 172**,** 109-122.

Munger, K. L., Levin, L. I., Hollis, B. W., Howard, N. S. & Ascherio, A. 2006. Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis. *Journal of the American Medical Association,* 296**,** 2832-2838.

Munger, K. L., Zhang, S. M., O'Reilly, E., Hernán, M. A., Olek, M. J., Willett, W. C. & Ascherio, A. 2004. Vitamin D intake and incidence of multiple sclerosis. *Neurology,* 62**,** 60-65.

Muramatsu, T., Tada, H., Kobayashi, N., Yamji, M., Shirai, T. & Ohnishi, T. 1992. Induction of the 72-kD heat shock protein in organ-cultured normal human skin. *J Invest Dermatol,* 98**,** 786-790.

Muramatsu, T., Yamashina, Y., Tada, H., Kobayashi, N., Yamaji, M., Ohno, H., Shirai, T., Takahashi, A. & Ohnishi, T. 1993. 8-methoxypsoralen plus UVA induces the 72 kDa heat shock protein in organ-cultured normal human skin. *Photochem Photobiol,* 58**,** 809-812.

Musiol, I. M. & Feldman, D. 1997. 1,25-dihydroxyvitamin D3 induction of nerve growth factor in L929 mouse fibroblasts: effect of vitamin D receptor regulation and potency of vitamin D3 analogs. *Endocrinology,* 138**,** 12-18.

Narita, M., Miyatake, M., Narita, M. & Shibasaki, M. 2006. Direct evidence of astrocytic modulation in the development of rewarding effects induced by drugs of abuse. *Neuropsychopharmacology,* 31**,** 2476-2488.

Nashold, F. E., Miller, D. J. & Hayes, C. E. 2000. 1,25-Dihydroxyvitamin D3 treatment decreases macrophage accumulation in the CNS of mice with experimental autoimmune encephalomyelitis. *Journal of Neuroimmunology,* 103**,** 171-179.

Nashold, F. E., Nelson, C. D., Brown, L. M. & Hayes, C. E. 2013. One calcitriol dose transiently increases Helios+FoxP3+ T cells and ameliorates autoimmune demyelinating disease. *Journal of Neuroimmunology,* 263**,** 64-74.

Nasti, T. H. & Timares, L. 2012. Inflammasome Activation of IL-1 Family Mediators in Response to Cutaneous Photodamage. *Photochemistry and Photobiology,* 88**,** 1111-1125.

Nataf, S., Garcion, E., Darcy, F., Chabannes, D., Muller, J. Y. & Brachet, P. 1996. 1,25 Dihydroxyvitamin D3 exerts regional effects in the central nervous system during experimental allergic encephalomyelitis. *J Neuropathol Exp Neurol,* 55**,** 904-914.

Nathiya, V. C. & Vanisree, A. J. 2010. Investigations on light induced stress model and on the role of Phyllanthus amarus in attenuation of stress related depression with focus on 5HT2Am- RNA expression. *Ann Neurosci,* 17**,** 167-175.

Naveilhan, P., Neveu, I., Wion, D. & Brachet, P. 1996. 1,25-Dihydroxyvitamin D3, an inducer of glial cell line-derived neurotrophic factor. *Neuroreport,* 7**,** 2171-2175.

Neveu, I., Naveilhan, P., Baudet, C., Brachet, P. & Metsis, M. 1994. 1,25-dihydroxyvitamin D3 regulates NT-3, NT-4 but not BDNF mRNA in astrocytes. *Neuroreport,* 6**,** 124-126.

Nolan, B. V. & Feldman, S. R. 2009. Ultraviolet tanning addiction. *Dermatologic clinics,* 27**,** 109-112.

Norman, A. W. 2008. From vitamin D to hormone D: fundamentals of the vitamin D endocrine system essential for good health. *Am J Clin Nutr,* 88**,** 491s-499s.

Northcutt, A. L., Hutchinson, M. R., Wang, X., Baratta, M. V., Hiranita, T., Cochran, T. A., Pomrenze, M. B., Galer, E. L., Kopajtic, T. A., Li, C. M., Amat, J., Larson, G., Cooper, D. C., Huang, Y., O'Neill, C. E., Yin, H., Zahniser, N. R., Katz, J. L., Rice, K. C., Maier, S. F., Bachtell, R. K. & Watkins, L. R. 2015. DAT isn't all that: cocaine reward and reinforcement require Toll-like receptor 4 signaling. *Molecular Psychiatry,* 20**,** 1525-1537.

Norval, M., McLoone, P., Lesiak, A. & Narbutt, J. 2008. The effect of chronic ultraviolet radiation on the human immune system. *Photochem Photobiol,* 84**,** 19-28.

Ojcius, D. M. & Saïd-Sadier, N. 2012. Alarmins, inflammasomes and immunity. *Biomedical Journal,* 35**,** 437-449.

Ousman, S. S. & Kubes, P. 2012. Immune surveillance in the central nervous system. *Nat Neurosci,* 15**,** 1096-1101.

Paillard, T., Rolland, Y. & de Souto Barreto, P. 2015. Protective Effects of Physical Exercise in Alzheimer's Disease and Parkinson's Disease: A Narrative Review. *J Clin Neurol,* 11**,** 212-219.

Pal, B. R., Marshall, T., James, C. & Shaw, N. J. 2003. Distribution analysis of vitamin D highlights differences in population subgroups: preliminary observations from a pilot study in UK adults. *J Endocrinol,* 179**,** 119-129.

Pedersen, L. B., Nashold, F. E., Spach, K. M. & Hayes, C. E. 2007. 1,25-Dihydroxyvitamin D3 reverses experimental autoimmune encephalomyelitis by inhibiting chemokine synthesis and monocyte trafficking. *Journal of Neuroscience Research,* 85**,** 2480-2490.

Petrulli, J. R., Kalish, B., Nabulsi, N. B., Huang, Y., Hannestad, J. & Morris, E. D. 2017. Systemic inflammation enhances stimulant-induced striatal dopamine elevation. *Translational psychiatry,* 7**,** 1-6.

Phillips, J. B., Jr. & Cox, B. M. 1997. Release of endogenous glutamate and gamma-aminobutyric acid from rat striatal tissue slices measured by an improved method of high performance liquid chromatography with electrochemical detection. *J Neurosci Methods,* 77**,** 211-220.

Pincelli, C. & Marconi, A. 2010. Keratinocyte stem cells: friends and foes. *J Cell Physiol,* 225**,** 310-315.

Poorsattar, S. P. & Hornung, R. L. 2007. UV light abuse and high-risk tanning behavior among undergraduate college students. *Journal of American Academy of Dermatology,* 56**,** 375-379.

Popoli, M., Yan, Z., McEwen, B. S. & Sanacora, G. 2011. The stressed synapse: the impact of stress and glucocorticoids on glutamate transmission. *Nat Rev Neurosci,* 13**,** 22-37.

Prietl, B., Treiber, G., Pieber, T. R. & Amrein, K. 2013. Vitamin D and immune function. *Nutrients,* 5**,** 2502-2521.

Proksch, E., Brandner, J. M. & Jensen, J. M. 2008. The skin: an indispensable barrier. *Exp Dermatol,* 17**,** 1063-1072.

Przedborski, S., Vila, M. & Jackson-Lewis, V. 2003. Neurodegeneration: what is it and where are we? *J Clin Invest,* 111**,** 3-10.

Quan, N. 2008. Immune-to-brain signaling: how important are the blood-brain barrier-independent pathways? *Mol Neurobiol,* 37**,** 142-152.

Quan, N. & Banks, W. A. 2007. Brain-immune communication pathways. *Brain, behavior, and immunity,* 21**,** 727-735.

Rahman, A. S., Pilver, C. E., Desai, R. A., Steinberg, M. A., Rugle, L., Krishnan-Sarin, S. & Potenza, M. N. 2012. The relationship between age of gambling onset and adolescent problematic gambling severity. *J Psychiatr Res,* 46**,** 675-683.

Randall, P. A., Pardo, M., Nunes, E. J., Lopez Cruz, L., Vemuri, V. K., Makriyannis, A., Baqi, Y., Muller, C. E., Correa, M. & Salamone, J. D. 2012. Dopaminergic modulation of effort-related choice behavior as assessed by a progressive ratio chow feeding choice task: pharmacological studies and the role of individual differences. *PLoS One,* 7**,** e47934.

Rapf, R. J. & Vaida, V. 2016. Sunlight as an energetic driver in the synthesis of molecules necessary for life. *Phys Chem Chem Phys,* 18**,** 20067-20084.

Rapuri, P. B., Gallagher, J. C. & Haynatzki, G. 2004. Effect of vitamins D2 and D3 supplement use on serum 25OHD concentration in elderly women in summer and winter. *Calcif Tissue Int,* 74**,** 150-156.

Raven, J. A., Cockell, C. S. & De La Rocha, C. L. 2008. The evolution of inorganic carbon concentrating mechanisms in photosynthesis. *Philos Trans R Soc Lond B Biol Sci,* 363**,** 2641-2650.

Robinson, J. K., Rigel, D. S. & Amonette, R. A. 1997. Trends in sun exposure knowledge, attitudes, and behaviors: 1986 to 1996. *Journal of the American Academy of Dermatology,* 37**,** 179-186.

Rock, K. L. & Kono, H. 2008. The inflammatory response to cell death. *Annu. Rev. pathmechdis. Mech. Dis.,* 3**,** 99-129.

Roh, B. H., Kim, D. H., Cho, M. K., Park, Y. L. & Whang, K. U. 2008. Expression of Heat Shock Protein 70 in Human Skin Cells as a Photoprotective Function after UV Exposure. *Ann Dermatol,* 20**,** 184-189.

Salamone, J. D. & Correa, M. 2012. The Mysterious Motivational Functions of Mesolimbic Dopamine. *Neuron,* 76**,** 470-485.

Salamone, J. D., Cousins, M. S. & Snyder, B. J. 1997. Behavioral functions of nucleus accumbens dopamine: empirical and conceptual problems with the anhedonia hypothesis. *Neurosci Biobehav Rev,* 21**,** 341-359.

Salamone, J. D., Wisniecki, A., Carlson, B. B. & Correa, M. 2001. Nucleus accumbens dopamine depletions make animals highly sensitive to high fixed ratio requirements but do not impair primary food reinforcement. *Neuroscience,* 105**,** 863-870.

Salmon, T., Evert, B., Song, B. & Doetsch, P. 2004. Biological consequences of oxidative stress-induced DNA damage in Saccharomyces cerevisiae. *Nucleic acids research,* 32**,** 3712-3723.

Samanek, A. J., Croager, E. J., Gies, P., Milne, E., Prince, R., McMichael, A. J., Lucas, R. M. & Slevin, T. 2006. Estimates of beneficial and harmful sun exposure times during the year for major Australian population centres. *Med J Aust,* 184**,** 338-341.

Sanchez, B., Relova, J. L., Gallego, R., Ben-Batalla, I. & Perez-Fernandez, R. 2009. 1,25-Dihydroxyvitamin D3 administration to 6-hydroxydopamine-lesioned rats increases glial cell line-derived neurotrophic factor and partially restores tyrosine hydroxylase expression in substantia nigra and striatum. *Journal of Neuroscience Research,* 87**,** 723-732.

Saper, C. B. & Breder, C. D. 1994. The neurologic basis of fever. *N Engl J Med,* 330**,** 1880-1886.

Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods,* 9**,** 671-675.

Schuch, A. P., Moreno, N. C., Schuch, N. J., Menck, C. F. M. & Garcia, C. C. M. 2017. Sunlight damage to cellular DNA: Focus on oxidatively generated lesions. *Free Radic Biol Med,* 107**,** 110-124.

Shaygannejad, V., Janghorbani, M., Ashtari, F. & Dehghan, H. 2012. Effects of adjunct low-dose vitamin d on relapsing-remitting multiple sclerosis progression: preliminary findings of a randomized placebo-controlled trial. *Mult Scler Int,* 2012**,** 7.

Shen, L. & Ji, H. F. 2015. Associations between vitamin D status, supplementation, outdoor work and risk of parkinson's disease: A meta-analysis assessment. *Nutrients,* 7**,** 4817-4827.

Silverman, A. J., Hoffman, D. L. & Zimmerman, E. A. 1981. The descending afferent connections of the paraventricular nucleus of the hypothalamus (PVN). *Brain Res Bull,* 6**,** 47-61.

Simpson Jr, S., Taylor, B., Blizzard, L., Ponsonby, A. L., Pittas, F., Tremlett, H., Dwyer, T., Gies, P. & Van Der Mei, I. 2010. Higher 25-hydroxyvitamin D is associated with lower relapse risk in multiple sclerosis. *Annals of Neurology,* 68**,** 193-203.

Sklar, L. R., Almutawa, F., Lim, H. W. & Hamzavi, I. 2012. Effects of ultraviolet radiation, visible light, and infrared radiation on erythema and pigmentation: a review. *Photochemical and Photobioligcal Sciences,* 12**,** 54-64.

Skobowiat, C., Dowdy, J. C., Sayre, R. M., Tuckey, R. C. & Slominski, A. 2011. Cutaneous hypothalamic-pituitary-adrenal axis homolog: regulation by ultraviolet radiation. *American journal of physiology. Endocrinology and metabolism,* 301**,** 93.

Skobowiat, C., Postlethwaite, A. E. & Slominski, A. T. 2017. Skin Exposure to Ultraviolet B Rapidly Activates Systemic Neuroendocrine and Immunosuppressive Responses. *Photochem Photobiol,* 93**,** 1008-1015.

Skobowiat, C. & Slominski, A. T. 2015. UVB Activates Hypothalamic-Pituitary-Adrenal Axis in C57BL/6 Mice. *J Invest Dermatol,* 135**,** 1638-1648.

Skobowiat, C. & Slominski, A. T. 2016. Ultraviolet B stimulates proopiomelanocortin signalling in the arcuate nucleus of the hypothalamus in mice. *Exp Dermatol,* 25**,** 120-123.

Sloka, S., Zhornitsky, S., Silva, C., Metz, L. M. & Yong, V. W. 2015. 1,25-Dihydroxyvitamin D3 Protects against Immune-Mediated Killing of Neurons in Culture and in Experimental Autoimmune Encephalomyelitis. *PLoS One,* 10**,** 11.

Slominski, A., Wortsman, J., Pisarchik, A., Zbytek, B., Linton, E. A., Mazurkiewicz, J. E. & Wei, E. T. 2001. Cutaneous expression of corticotropin-releasing hormone (CRH), urocortin, and CRH receptors. *Faseb j,* 15**,** 1678-1693.

Slominski, A. T. 2015. Ultraviolet radiation (UVR) activates central neuro-endocrine-immune system. *Photodermatology, photoimmunology & photomedicine,* 31**,** 121-123.

Slominski, A. T., Zmijewski, M. A., Plonka, P. M., Szaflarski, J. P. & Paus, R. 2018. How UV Light Touches the Brain and Endocrine System Through Skin, and Why. *Endocrinology,* 159**,** 1992-2007.

Slominski, A. T., Zmijewski, M. A., Zbytek, B., Tobin, D. J., Theoharides, T. C. & Rivier, J. 2013. Key role of CRF in the skin stress response system. *Endocr Rev,* 34**,** 827-884.

Smith, M. P., Fletcher-Turner, A., Yurek, D. M. & Cass, W. A. 2006. Calcitriol protection against dopamine loss induced by intracerebroventricular administration of 6-hydroxydopamine. *Neurochem Res,* 31**,** 533-539.

Soilu-Hänninen, M., Åivo, J., Lindström, B. M., Elovaara, I., Sumelahti, M. L., Färkkilä, M., Tienari, P., Atula, S., Sarasoja, T., Herrala, L., Keskinarkaus, I., Kruger, J., Kallio, T., Rocca, M. A. & Filippi, M. 2012. A randomised, double blind, placebo controlled trial with vitamin D 3 as an add on treatment to interferon β-1b in patients with multiple sclerosis. *Journal of Neurology, Neurosurgery and Psychiatry,* 83**,** 565-571.

Soleimani, M., Jameie, S. B., Mehdizadeh, M., Keradi, M., Masoumipoor, M. & Mehrabi, S. 2014. Vitamin D3 influence the Th1/Th2 ratio in C57BL/6 induced model of experimental autoimmune encephalomyelitis. *Iranian Journal of Basic Medical Sciences,* 17**,** 785-792.

Solomon, J. A., Gianforcaro, A. & Hamadeh, M. J. 2011. Vitamin D3 deficiency differentially affects functional and disease outcomes in the G93A mouse model of amyotrophic lateral sclerosis. *PLoS One,* 6**,** e29354.

Spach, K. M. & Hayes, C. E. 2005. Vitamin D3 confers protection from autoimmune encephalomyelitis only in female mice. *Journal of Immunology,* 175**,** 4119-4126.

Spach, K. M., Nashold, F. E., Dittel, B. N. & Hayes, C. E. 2006. IL-10 signaling is essential for 1,25-dihydroxyvitamin D 3-mediated inhibition of experimental autoimmune encephalomyelitis. *Journal of Immunology,* 177**,** 6030-6037.

Stapleton, J. L., Hillhouse, J., Levonyan-Radloff, K. & Manne, S. L. 2017. Review of interventions to reduce ultraviolet tanning: Need for treatments targeting excessive tanning, an emerging addictive behavior. *Psychol Addict Behav,* 31**,** 962-978.

Stein, M. S., Liu, Y., Gray, O. M., Baker, J. E., Kolbe, S. C., Ditchfield, M. R., Egan, G. F., Mitchell, P. J., Harrison, L. C., Butzkueven, H. & Kilpatrick, T. J. 2011a. A randomized trial of high-dose vitamin D2 in relapsing-remitting multiple sclerosis. *Neurology,* 77**,** 1611-1618.

Stein, M. S., Scherer, S. C., Ladd, K. S. & Harrison, L. C. 2011b. A randomized controlled trial of high-dose vitamin D2 followed by intranasal insulin in Alzheimer's disease. *J Alzheimers Dis,* 26**,** 477-484.

Stellwagen, D., Beattie, E. C., Seo, J. Y. & Malenka, R. C. 2005. Differential regulation of AMPA receptor and GABA receptor trafficking by tumor necrosis factor-alpha. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 25**,** 3219-3228.

Stevens, C. W., Aravind, S., Das, S. & Davis, R. L. 2013. Pharmacological characterization of LPS and opioid interactions at the toll like receptor 4. *British journal of pharmacology,* 168**,** 1421-1429.

Stocklin, E. & Eggersdorfer, M. 2013. Vitamin D, an essential nutrient with versatile functions in nearly all organs. *Int J Vitam Nutr Res,* 83**,** 92-100.

Sulzer, D. 2011. How addictive drugs disrupt presynaptic dopamine neurotransmission. *Neuron,* 69**,** 628-649.

Suzuki, M., Yoshioka, M., Hashimoto, M., Murakami, M., Noya, M., Takahashi, D. & Urashima, M. 2013. Randomized, double-blind, placebo-controlled trial of vitamin D supplementation in Parkinson disease. *Am J Clin Nutr,* 97**,** 1004-1013.

Swann, G. 2010. The skin is the body's largest organ. *J Vis Commun Med,* 33**,** 148-149.

Taghizadeh, M., Djazayery, A., Salami, M., Eshraghian, M. R. & Zavareh, S. A. 2011. Vitamin-D-free regimen intensifies the spatial learning deficit in Alzheimer's disease. *Int J Neurosci,* 121**,** 16-24.

Taghizadeh, M., Talaei, S. A., Djazayeri, A. & Salami, M. 2014. Vitamin D supplementation restores suppressed synaptic plasticity in Alzheimer's disease. *Nutr Neurosci,* 17**,** 172-177.

Takai, E., Tsukimoto, M., Harada, H. & Kojima, S. 2011. Involvement of P2Y6 receptor in p38 MAPK-mediated COX-2 expression in response to UVB irradiation of human keratinocytes. *Radiat Res,* 175**,** 358-366.

Tarbali, S. & Khezri, S. 2016. Vitamin D3 attenuates oxidative stress and cognitive deficits in a model of toxic demyelination. *Iranian Journal of Basic Medical Sciences,* 19**,** 80-88.

Taylor, C. L., Sempos, C. T., Davis, C. D. & Brannon, P. M. 2017. Vitamin D: Moving Forward to Address Emerging Science. *Nutrients,* 9.

Thacher, T. D. & Clarke, B. L. 2011. Vitamin D insufficiency. *Mayo Clinic Proceedings,* 86**,** 50-60.

Titus, J. C., Godley, S. H. & White, M. K. 2007. A post-treatment examination of adolescents' reasons for starting, quitting, and continuing the use of drugs and alcohol. *Journal of Child & Adolescent Substance Abuse,* 16**,** 31-49.

Trang, H. M., Cole, D. E., Rubin, L. A., Pierratos, A., Siu, S. & Vieth, R. 1998. Evidence that vitamin D3 increases serum 25-hydroxyvitamin D more efficiently than does vitamin D2. *Am J Clin Nutr,* 68**,** 854-858.

Trautinger, F., Trautinger, I., Kindas-Mugge, I., Metze, D. & Luger, T. A. 1993. Human keratinocytes in vivo and in vitro constitutively express the 72-kD heat shock protein. *J Invest Dermatol,* 101**,** 334-338.

Tsan, M.-F. & Gao, B. 2004. Endogenous ligands of Toll-like receptors. *Journal of leukocyte biology,* 76**,** 514-519.

Tschopp, J. & Schroder, K. 2010. NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production? *Nature Reviews Immunology,* 10**,** 210-215.

Tyrrell, R. 1995. Ultraviolet radiation and free radical damage to skin. *Biochemical Society Symposia,* 61**,** 47-53.

Tyrrell, R. M. 2000. Role for singlet oxygen in biological effects of ultraviolet A radiation. *Methods Enzymol,* 319**,** 290-296.

Van Dam, A. M., Brouns, M., Man, A. H. W. & Berkenbosch, F. 1993. Immunocytochemical detection of prostaglandin E2 in microvasculature and in neurons of rat brain after administration of bacterial endotoxin. *Brain Res,* 613**,** 331-336.

Van Der Mei, I. A., Ponsonby, A. L., Dwyer, T., Blizzard, L., Taylor, B. V., Kilpatrick, T., Butzkueven, H. & McMichael, A. J. 2007. Vitamin D levels in people with multiple sclerosis and community controls in Tasmania, Australia. *J Neurol,* 254**,** 581-590.

Van Der Mei, I. A. F., Ponsonby, A. L., Dwyer, T., Blizzard, L., Simmons, R., Taylor, B. V., Butzkueven, H. & Kilpatrick, T. 2003. Past exposure to sun, skin phenotype, and risk of multiple sclerosis: Case-control study. *British Medical Journal,* 327**,** 316-320.

van Schoor, N. & Lips, P. 2017. Global Overview of Vitamin D Status. *Endocrinol Metab Clin North Am,* 46**,** 845-870.

Videira, I. F. d. S., Moura, D. F. L. & Magina, S. 2013. Mechanisms regulating melanogenesis. *Anais brasileiros de dermatologia,* 88**,** 76-83.

Viviani, B., Boraso, M., Marchetti, N. & Marinovich, M. 2014. Perspectives on neuroinflammation and excitotoxicity: a neurotoxic conspiracy? *Neurotoxicology,* 43**,** 10-20.

Volkow, N. D. & McLellan, A. T. 2016. Opioid abuse in chronic pain—misconceptions and mitigation strategies. *New England Journal of Medicine,* 374**,** 1253-1263.

Wacker, M. & Holick, M. F. 2013. Sunlight and Vitamin D: A global perspective for health. *Dermatoendocrinol,* 5**,** 51-108.

Wang, J., Yang, D., Yu, Y., Shao, G. & Wang, Q. 2016. Vitamin D and Sunlight Exposure in Newly-Diagnosed Parkinson's Disease. *Nutrients,* 8**,** 142.

Wang, J. Y., Wu, J. N., Cherng, T. L., Hoffer, B. J., Chen, H. H., Borlongan, C. V. & Wang, Y. 2001. Vitamin D(3) attenuates 6-hydroxydopamine-induced neurotoxicity in rats. *Brain Res,* 904**,** 67-75.

Wang, L. Y., Evatt, M. L., Maldonado, L. G., Perry, W. R., Ritchie, J. C., Beecham, G. W., Martin, E. R., Haines, J. L., Pericak-Vance, M. A., Vance, J. M. & Scott, W. K. 2015a. Vitamin D From Different Sources Is Inversely Associated With Parkinson Disease. *Movement Disorders,* 30**,** 560-566.

Wang, Y., Marling, S. J., Beaver, E. F., Severson, K. S. & Deluca, H. F. 2015b. UV light selectively inhibits spinal cord inflammation and demyelination in experimental autoimmune encephalomyelitis. *Archives of Biochemistry and Biophysics,* 567**,** 75-82.

Wang, Y., Marling, S. J., Zhu, J. G., Severson, K. S. & DeLuca, H. F. 2012. Development of experimental autoimmune encephalomyelitis (EAE) in mice requires vitamin D and the vitamin D receptor. *Proceedings of the National Academy of Sciences of the United States of America,* 109**,** 8501-8504.

Wang, Y. P., Marling, S. J., McKnight, S. M., Danielson, A. L., Severson, K. S. & Deluca, H. F. 2013. Suppression of experimental autoimmune encephalomyelitis by 300-315 nm ultraviolet light. *Archives of Biochemistry and Biophysics,* 536**,** 81-86.

Warthan, M. M., Uchida, T. & Wagner, R. F. 2005. UV Light Tanning as a Type of Substance-Related Disorder. *Archives of Dermatology,* 141**,** 963-966.

Watkins, L. R. & Maier, S. F. 2000. The pain of being sick: implications of immune-to-brain communication for understanding pain. *Annu Rev Psychol,* 51**,** 29-57.

Willemse, S. & Furger, M. 2016. *From weather observations to atmospheric and climate sciences in Switzerland. Celebrating 100 years of the Swiss Society for Meteorology*, vdf Hochschulverlag AG an der ETH Zürich.

Wong, D., Prameya, R. & Dorovini-Zis, K. 2007. Adhesion and migration of polymorphonuclear leukocytes across human brain microvessel endothelial cells are differentially regulated by endothelial cell adhesion molecules and modulate monolayer permeability. *Journal of neuroimmunology,* 184**,** 136-148.

Yohn, S. E., Santerre, J. L., Nunes, E. J. & Kozak, R. 2015. The role of dopamine D1 receptor transmission in effort-related choice behavior: Effects of D1 agonists. *Pharmacology, Biochemistry and Behaviour,* 135**,** 217-226.

Yoshizumi, M., Nakamura, T., Kato, M., Ishioka, T., Kozawa, K., Wakamatsu, K. & Kimura, H. 2008. Release of cytokines/chemokines and cell death in UVB-irradiated human keratinocytes, HaCaT. *Cell Biology International,* 32**,** 1405-1411.

Young, A. R. 1997. Chromophores in human skin. *Phys Med Biol,* 42**,** 789-802.

Young, C. 2009. Solar ultraviolet radiation and skin cancer. *Occupational Medicine,* 59**,** 82-88.

Yu, J., Gattoni-Celli, M., Zhu, H., Bhat, N. R., Sambamurti, K., Gattoni-Celli, S. & Kindy, M. S. 2011. Vitamin D3-enriched diet correlates with a decrease of amyloid plaques in the brain of AbetaPP transgenic mice. *J Alzheimers Dis,* 25**,** 295-307.

Zalcman, S., Green-Johnson, J. M., Murray, L., Nance, D. M., Dyck, D., Anisman, H. & Greenberg, A. H. 1994. Cytokine-specific central monoamine alterations induced by interleukin-1, -2 and -6. *Brain Res,* 643**,** 40-49.

Zeitelhofer, M., Adzemovic, M. Z., Gomez-Cabrero, D., Bergman, P., Hochmeister, S., N'Diaye, M., Paulson, A., Ruhrmann, S., Almgren, M., Tegner, J. N., Ekstrom, T. J., Guerreiro-Cacais, A. O. & Jagodic, M. 2017. Functional genomics analysis of vitamin D effects on CD4+ T cells in vivo in experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A,* 114**,** E1678-e1687.

Zeller, S., Lazovich, D. A., Forster, J. & Widome, R. 2006. Do adolescent indoor tanners exhibit dependency? *Journal of American Academy of Dermatology,* 54**,** 589-596.

Zhang, M., Balmadrid, C. & Kelley, A. E. 2003. Nucleus accumbens opioid, GABaergic, and dopaminergic modulation of palatable food motivation: contrasting effects revealed by a progressive ratio study in the rat. *Behav Neurosci,* 117**,** 202-211.

Zhang, R. & Naughton, D. P. 2010. Vitamin D in health and disease: Current perspectives. *Nutrition Journal,* 9**,** 13.

Zhao, H., Traganos, F. & part A, D.-Z. 2010. Kinetics of the UV-induced DNA damage response in relation to cell cycle phase. Correlation with DNA replication. *Cytometry part A: : the journal of the International Society for Analytical Cytology* 77**,** 285-293.

Zheng, C., He, L., Liu, L., Zhu, J. & Jin, T. 2018. The efficacy of vitamin D in multiple sclerosis: A meta-analysis. *Mult Scler Relat Disord,* 23**,** 56-61.

Zhu, D., Liu, G. Y., Lv, Z., Wen, S. R., Bi, S. & Wang, W. Z. 2014. Inverse associations of outdoor activity and vitamin D intake with the risk of Parkinson's disease. *J Zhejiang Univ Sci B,* 15**,** 923-927.

Zhu, H., Wang, N., Yao, L., Chen, Q., Zhang, R., Qian, J., Hou, Y., Guo, W., Fan, S., Liu, S., Zhao, Q., Du, F., Zuo, X., Guo, Y., Xu, Y., Li, J., Xue, T., Zhong, K., Song, X., Huang, G. & Xiong, W. 2018. Moderate UV Exposure Enhances Learning and Memory by Promoting a Novel Glutamate Biosynthetic Pathway in the Brain. *Cell,* 173**,** 1716-1727.e1717.

Zmijewski, M. A. & Slominski, A. T. 2011. Neuroendocrinology of the skin: An overview and selective analysis. *Dermatoendocrinol,* 3**,** 3-10.

Appendices

- **Appendix A:** Design of a novel apparatus for the safe delivery of targeted UVR on the skin
- **Appendix B**: Pilot study results
- **Appendix C:** Source code written for operant conditioning boxes

The proposed study design described in Chapter 3 reflects a body of work that was planned with the intention of designing a new experimental protocol for the safe and targeted administration of UV light to animals; to test potential motivational properties associated with exposure. This included the comprehensive design of a new apparatus that enabled plasma light to be administered directly onto an area of the skin via a fibre optic cable, and extensive research and planning of experimental protocols to address the research questions described in section 3.2. However, during initial pilot tests and training phases the candidate and lead investigator experienced a serious injury that had ongoing complications, significantly impacting on progression of the research. The uncertainty surrounding the longevity of personal treatment and unpredictable circumstances ultimately restricted the capacity of completing the research goals within the given time frame of candidature. Thus, the research focus was shifted away from the proposed study design and the project was not completed. Detailed in the Appendices is the design of the UV light apparatus, protocols and preliminary results from initial pilot tests, and the source codes that were developed for behavioural testing. Together demonstrating the foundational work that had been underway prior to injury.

Appendix A: Design of novel apparatus for the delivery of targeted ultraviolet radiation on the skin

As detailed in section 3.7.2, a novel UV light apparatus was developed that guides UVR directly onto a target area of skin. In this design, UV light is emitted from a plasma light source and then travels along a fibre optic cable connected to a titanium dispersing cone that is worn by the animal (displayed in Figure A.1). Designing a secure-fitting UV light apparatus was paramount for the outlined research project as it enables precise quantities of UVR to be applied to a designated area. This serves to remove ambiguity surrounding the amount of radiation received by the animal as no radiance is lost, thus providing greater clarity of subtle differences in the physiological response to a range of UVR intensities. Additionally, reducing the UVR exposure to a targeted area of the skin eliminates unnecessary exposure to the eyes, ears and paws, minimising harm to highly sensitive tissues. Furthermore, containing the light within the dispersing cone eliminates a potential stress response triggered by an inescapable bright light (Barker et al., 2010) and any associated harm which could significantly impact on results.

The dispersing cone (Figure A.2A) was designed modelling the curvature of a Sprague Dawley rat, and using titanium, was 3D-printed by the Australian National Fabrication Facility at the University of Adelaide. UVR was generated with an EQ-99 laser-driven light source (Energetiq Technology Inc., Wilmington, MA, United States) known to produce high brightness, broad band light. The emission spectrum captured (Figure A.2B) resembles solar emission spectrum recorded at sea level including light wavelengths from ~290nm to above 900nm. A filter blocking all wavelengths below 400nm facilitated sham UVR for experimental purposes.

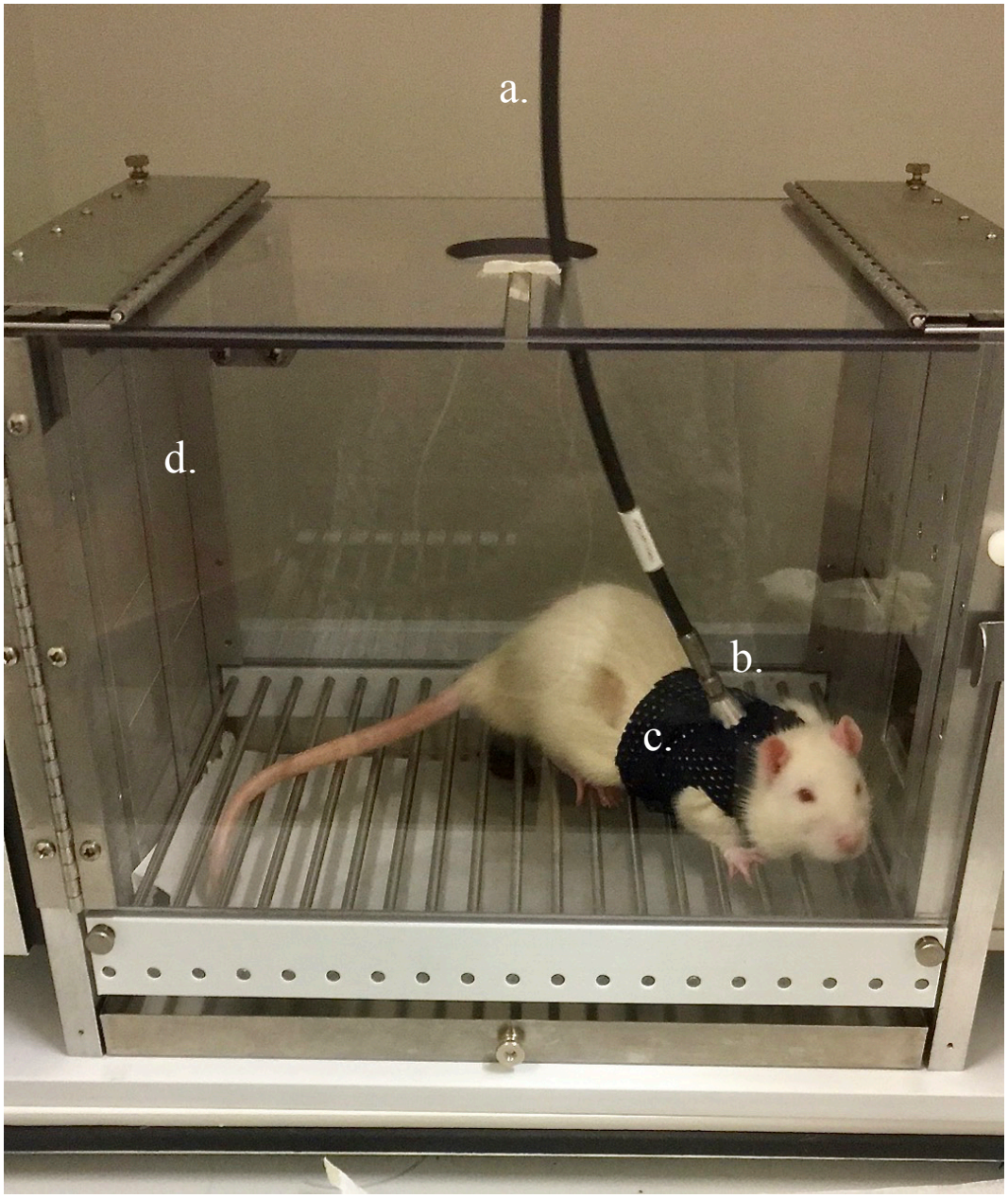


Figure A.1. Photograph of the UV light administration using the novel apparatus. UV light from a plasma light source (not shown) is delivered to the scapular region of the animal along a fiber optic cable (a.). The cable connects to a light weight titanium dispersing cone (b) that is sits within a material vest, worn by the animal (c.). All experimental procedures were performed within an operant conditioning chamber (d.).

A.

B.

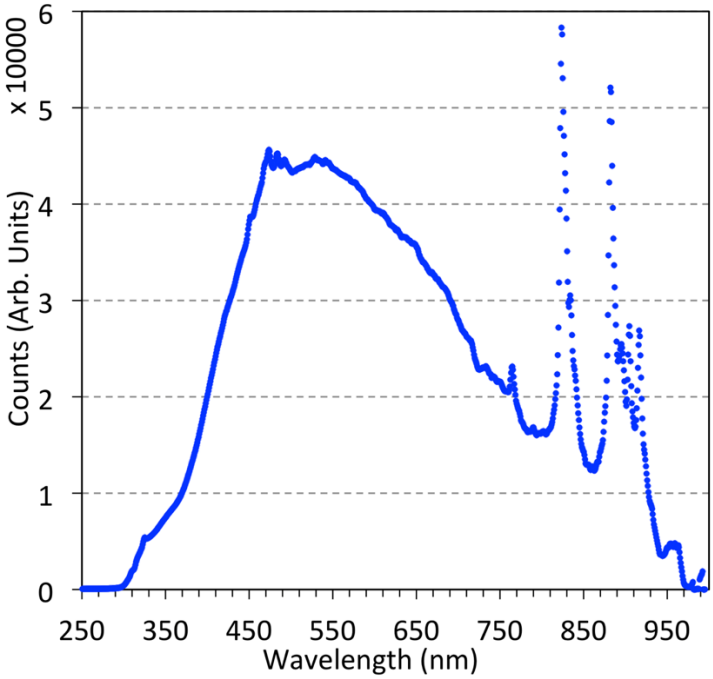


Figure A.2. (A.) Image of the dispersing cone designed to be worn by the animals for the administration of UVR. (B.) The output spectra of wavelength emissions from the UV light apparatus. Demonstrating that the spectra of light emitted is comparable to solar emissions recorded at sea level.

Appendix B: Pilot results

Pilot 1A: Acute ultraviolet exposure to assess skin response

The UV light apparatus employs a new method for delivery of UVR, therefore a pilot study was undertaken to assess different levels of exposure (amount of UVR received) in an acute setting. This was necessary to ensure the light apparatus was effective, without causing excessive skin damage and to test study protocols. Animals were exposed to either 0, 2 or 4 kj/m^2 of UVR and then tissues were collected four hours post-exposure. Histological changes in the skin were assessed with routine H&E staining. This pilot study also provided the opportunity to test the backpack design for fitting the dispersing cone to the scapular region of the animal.

UVR exposure

- 0 kj/m^2 - *equating 1800s (30m) of sham UVR*
- 2 kj/m^2 - *equating to 868s (14.4m) of UVR and 939s (15.6m) of sham UVR*
- 4 kj/m^2 - *equating to 1736s (28.9m) of UVR and 66s (1.1m) of sham UVR*

Animals: N=9 (3 per group)

- Tissue collection +4 hours post UVR to capture mid-range of the expected 2-6 peak elevation of alarmin/inflammatory mediators (Catania et al., 1999; Peltz et al., 2009).

Protocol

Day 1.

1. Anesthetise animal using isoflurane inhalation (1.5-2% in O^2)
2. Shave scapular region with clippers
3. Tail-vein blood collection

Day 2.

1. Ensure UV light barrier is in place
2. Turn on light source, cooling fan and operant box
3. Collect animals from home cages, as per experiment guide
4. Fit animal with light cone apparatus and position within operant box, attaching fibre cable to the dispersing cone
5. Double check animal ID and exposure time with experiment guide
6. Remove UVR filter (if applicable) set timers (UVR timer and 30-minute test timer) and simultaneously remove light barrier.
7. Once UVR timer has elapsed, apply filter for remainder of test
8. At 29 minutes (after UVR filter has been applied) save spectrum analysis
9. Block light source with barrier
10. Disconnect fibre, remove UV apparatus and return animal to home cage.
11. Set a 4-hour timer for post exposure tissue collection
12. Clean operant box for next use

13. Set up work bench for cardiac perfusion, tissue dissection and collection *(saline, ice, labelled tubes, liquid nitrogen)*
14. Anaesthetise animal using isoflurane
15. Perform cardiac bleed and store blood in EDTA tubes
16. Transcardial saline perfusion until tissues are clear of blood
17. Collect brain, spinal cord and skin tissue (exposed and unexposed site) and place in labelled tubes.
    a. Half of skin samples to be placed in 10% formalin for histology
18. Freeze tissue immediately in liquid nitrogen

Histological analysis with H&E

Skin samples from the irradiated site and a proximal non-irradiated site were prepared for histological assessment following the protocol described in section 4.5.5. Scanned image files were analysed using ImageJ software and assessed for:

a. Cell morphology (length of basal keratinocytes)
b. Epithelial layer thickness (as a portion of total thickness)
c. Layer intensity (i.e. were cells becoming more compressed due to the UV light and therefore increasing the intensity (number of pixels) within a set parameter from each layer)

To facilitate analysis, images were converted to 8bit, black and white images, and then a standardised "region of interest" was selected to obtain measurements of pixel intensity and quantity within each region. Comparisons were then made between the UVR exposure site and the non-UVR exposure site for each animal and between different exposure groups (Figure B.1). Despite extensive analysis, no significant differences in all measures between the UVR and non-UVR tissue or UVR quantity were found (Figures B.2-B.4). Leading to the conclusion that a single acute exposure of UVR at 0, 2 or 4 kj/m^2 in this model, was not inducing detectable histological changes in the skin. This was consistent with visual assessment of the skin tissue immediately after exposure and at four hours post-exposure, where no visible differences were detected.

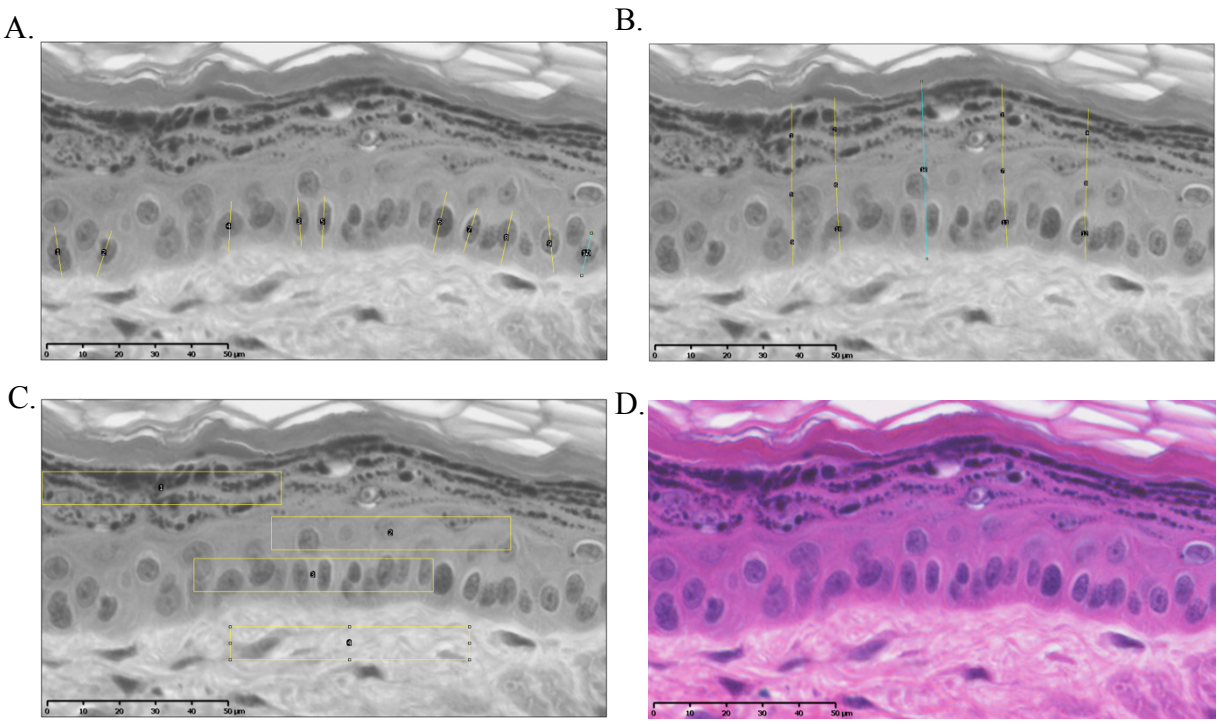


Figure B.1. Histological assessment acute UVR study. (A.) Cell length (upwards from the basement membrane) was recorded from 10 individual cells per image to detect if UVR contributed to alterations in keratinocyte morphology. (B.) Analysis of layer thickness by measuring three adjacent regions of the epidermis the stratum basale, stratum spinosum and stratum granulosum (as a proportion to total thickness) to determine if UVR contributed to layer differences. (C.) Layer intensity was measured by looking at the ratio of light/dark pixels of each layer, including the dermis, to detect a potential shift in density induced by UVR. (D.) Original image showing H&E staining**.**

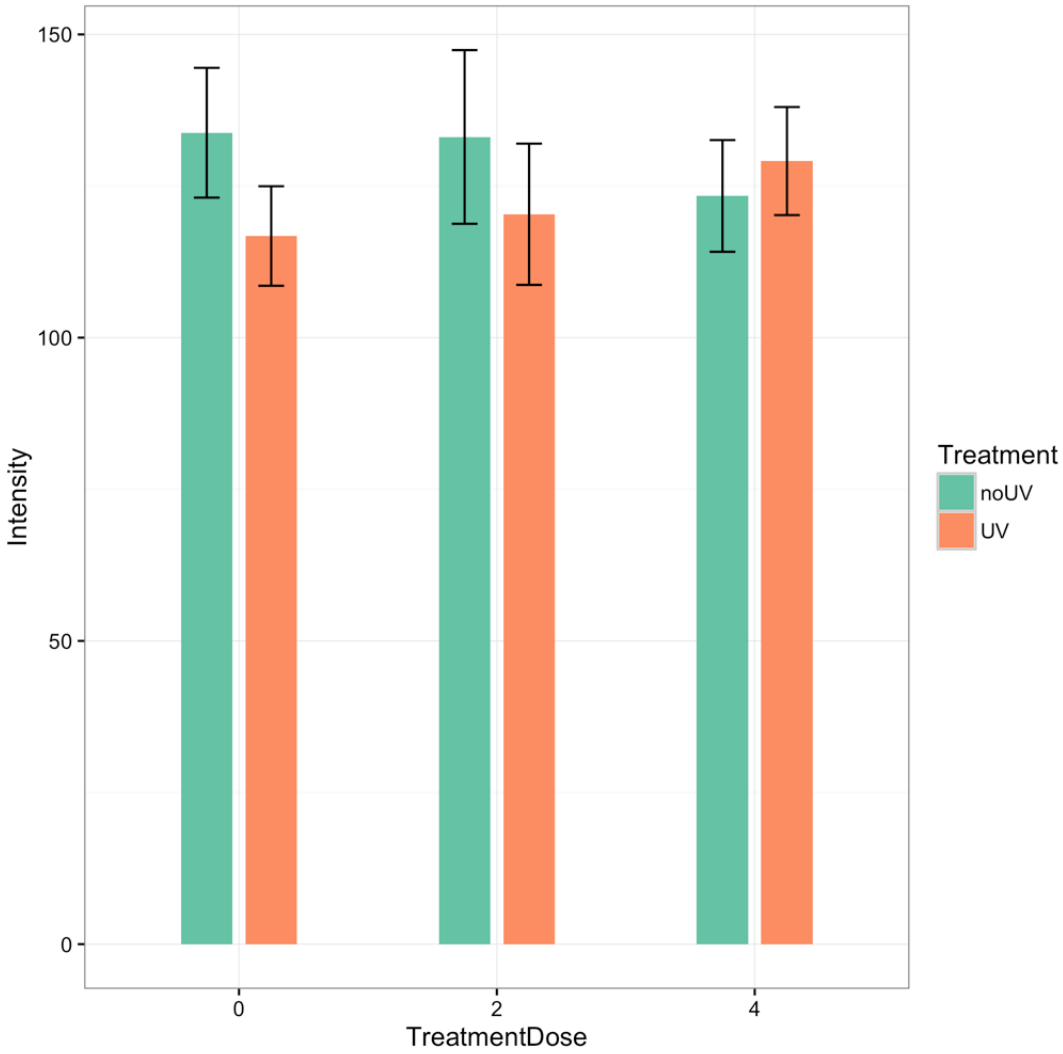


Figure B.2. Analysis of cell morphology of basale keratinocytes. Skin samples were collected from the irradiated site and a proximal non-irradiated site, following exposure with either 0, 2 or 4 kj/m^2 of UVR (n=3 per group). Measurements of basale keratinocytes length (upwards from the basement membrane) were then compared between groups to detect UVR-related changes. Statistical significance was not reached ($p>0.05$). Statistical calculations were performed using liner mixed-effects model fit from R software (R Core team, 2016) comparing the mean intensity (length of cell) with the treatment dose.

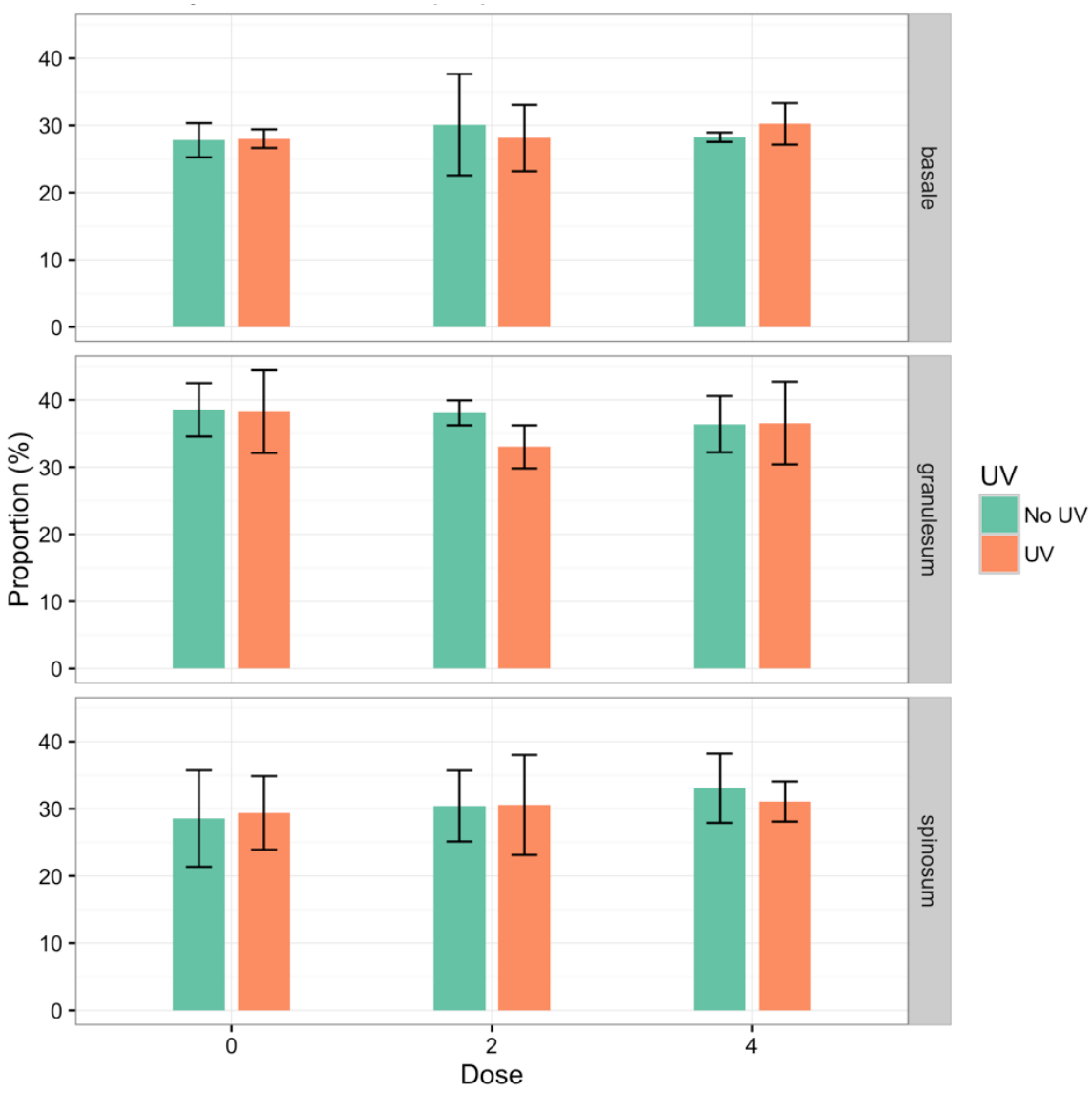


Figure B.3. Layer thickness as proportion of total thickness. Skin samples were collected from the irradiated site and a proximal non-irradiated site, following exposure with either 0, 2 and 4 kj/m^2 of UVR (n=3 per group). The proportional length of the basale, granulosum and spinosum layers of the epidermis were determined from the samples collected. An ANOVA with Tukey multiple comparisons was performed using GraphPad Prism 7 software (GraphPad Software Inc.; California, USA). Statistical significance was not found ($p>0.05$).

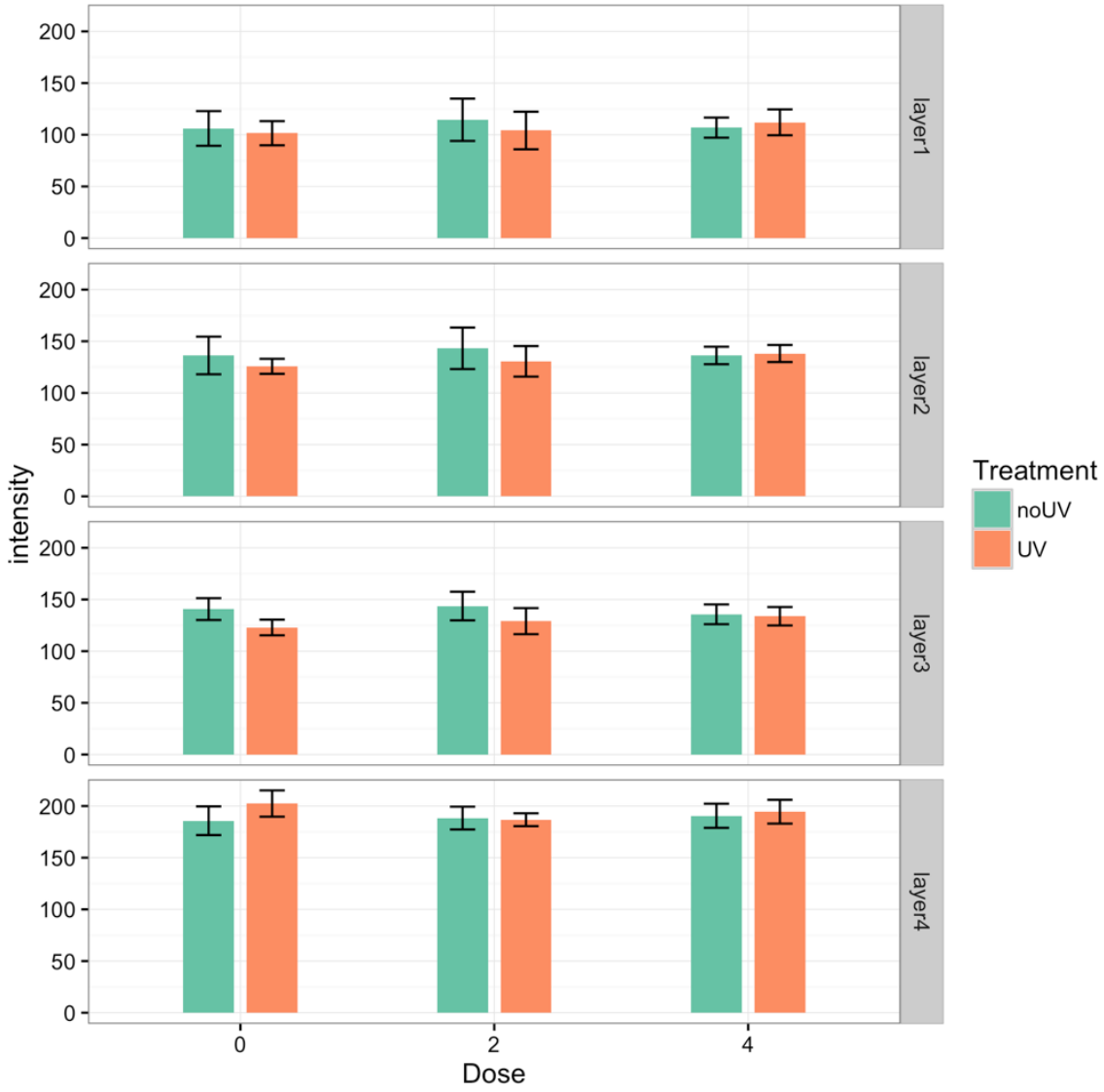


Figure B.4. Layer intensity. Skin samples were collected from the irradiated site and a proximal non-irradiated site, following exposure with either 0, 2 or 4 kj/m^2 of UVR (n=3 per group). A standardised area was measured within each layer of skin tissue, representing the stratum granulosum (layer 1), the stratum spinosum (layer 2), the stratum basale (layer 3) and the dermis (layer 4) from which the ratio of light/dark pixels were compared to detect a potential shift in density (increased dark pixels) induced by UVR. No significant detectable changes were observed in any layer between the UVR and the sham UVR exposed skin.

Outcomes

1. This method of UVR administration, at physiologically relevant doses, was not causing harm/distress to the animals.
2. A single cutaneous dose of UVR did not induce changes to skin tissue, using H&E analysis, four-hours following UVR exposure.
3. Re-design of the backpack is necessary to ensure secure fitting of the dispersing cone to the animal.

Pilot 1B: Repeated ultraviolet exposure to assess skin response after 7 days

Following the acute pilot study, increased UVR exposure, over consecutive days was undertaken aiming to elicit detectable UVR-induced changes to skin tissue. This study followed the same protocol as the acute study, however exposure was repeated for 7 days. Tissue was collected four-hours post exposure on day 7. Only 0 and 4 kj/m^2 of UVR exposures were used to allow for larger group sizes. Tissue analysis was performed using HMGB1 antibody staining, as HMGB1 is a prominent alarmin released from cells during times of injury.

UVR exposure

- 0 kj/m^2 - *equating 1800s (30m) of sham UVR*
- 4 kj/m^2 - *equating to 1736s (28.9m) of UVR and 66s (1.1m) of sham UVR*

Animals: N=9 (4-5 per group)

- Tissue collection +4 hours post UVR exposure

Protocol

This study followed the same protocol as the acute UVR study repeating "day 2" procedures for seven consecutive days. There was a minor amendment in that the spectrum

analysis was recorded at two time points, an initial recording prior to removing the UVR filter and then again at the end of the test. The dispersing cone was also more securely fitted to the animal with the new fabric vest design as displayed in Appendix A.

Histological tissue analysis with H&E

Skin samples were once again collected as described in section 4.5.5 and were stained with routine H&E, as in the acute study. However, as there were no evident differences between each site (UVR and non-UVR areas) and between UVR and sham UVR groups detected during preliminary screening, no further analysis of H&E slides was performed and H&E staining was determined insufficient to detect variance in these samples.

Immunohistochemistry

Immunohistochemistry was performed to analyse the presence of the alarmin HMGB1 in skin tissue following UVR and sham UVR exposure. HMGB1 was expected to be downregulated in the UVR treated animals, therefore reduced HMGB1 staining in the UVR tissue, as there would be less HMGB1 in the tissue once it was released from damaged skin cells. A general immunohistochemistry staining procedure has been detailed in section 4.5.6. Briefly, antigen retrieval was completed using citrate buffer and endogenous peroxidase activity blocked with 3% hydrogen peroxide in methanol. Non-specific binding was blocked using 10% normal horse serum in phosphate buffered saline (PBS). Slides were then incubated overnight at room temperature in primary antibodies for HMGB1 (Abcam #Ab19256 1:5000). Sections were washed in PBS and incubated in a corresponding biotinylated secondary antibody for 60 minutes and then incubated in avidin horseradish peroxidase solution for 60 minutes. Staining was developed with 3,3’ Diaminobenzidine (DAB) and sections were washed in running water before counterstaining with hematoxylin, and coverslips applied.

Scanned slides were initially assessed using the DAB colour deconvolution function in Image J Fiji software to calculate the optical density (max intensity/mean intensity) of the DAB separated file image. Optical density analysis was performed on the epidermis and dermis together, the epidermis only and the dermis only, however, significant differences between the exposure site and not-exposure sites, as well as between UVR and sham UVR was not detected (Figure B5).

Next, manual cell counts of all positively stained cells in either the epidermis, dermis (portion of the dermis just below the epidermis) and sub dermis (remaining portion of the dermis) regions were obtained to detect possible differences in the number of cells showing HMGB1 staining. Image files were blinded and the average number of cells per μm^2 were determined. Cell counts from each area were then compared between exposure groups (Figure B6). However, no significant differences were detected.

In a final effort to capture an effect, semi-quantitative graded analysis was performed on both the epidermal and dermal sections of tissue. For this analysis, blinded images were assessed by three independent persons and staining intensity was graded as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. Any discrepancies in grading were discussed until a consensus was reached. Still, a significant effect was not distinguishable between groups (data not shown).

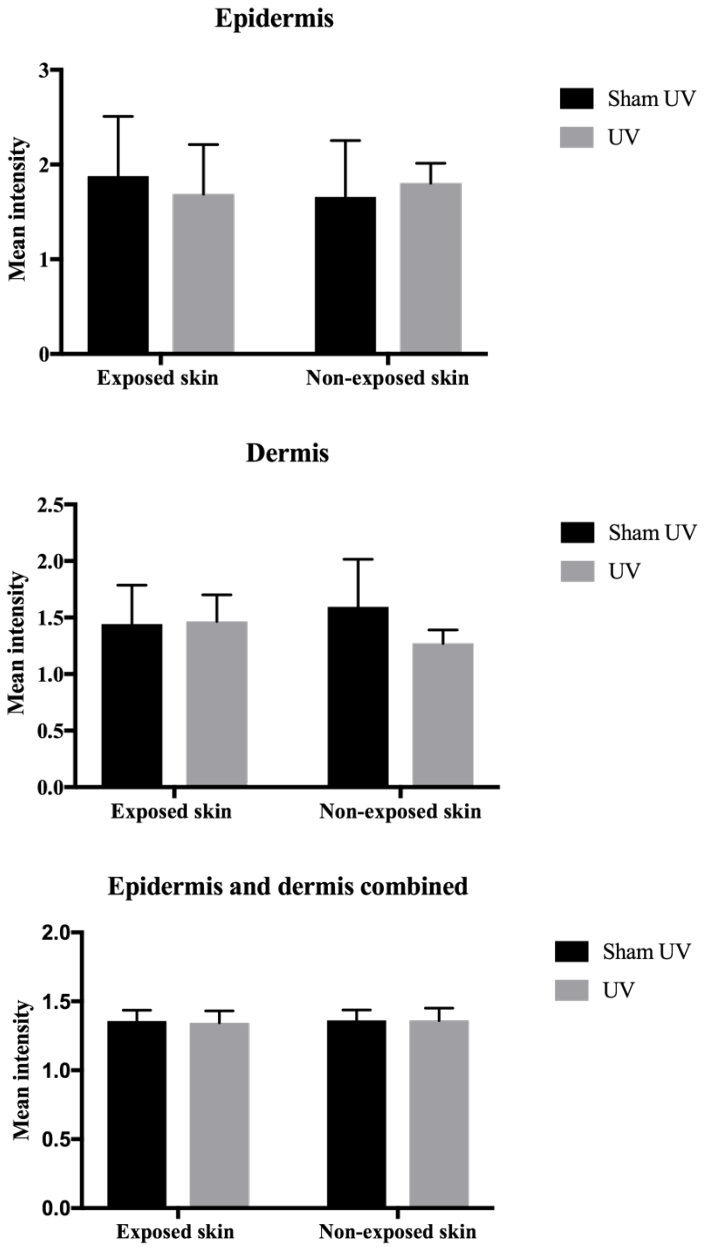


Figure B5. Optical density of HMGB1 staining using DAB colour deconvolution function in Fiji ImageJ software. Skin samples were collected from the irradiated site and a proximal non-irradiated site, following exposure with either 0 (sham UVR) or 4 kj/m^2 of UVR (n=3-5 per group). Colour deconvolution allows separation of an image file into channels, so the intensity of DAB staining can be assessed within a selected area. GraphPad prism software 7 **(**GraphPad Software Inc.; California, USA) was used to perform a two-way ANOVA to analyse the mean intensity of the DAB stain within the epidermis, dermis and the two regions combined, yet, results were non-significant ($p>0.05$).

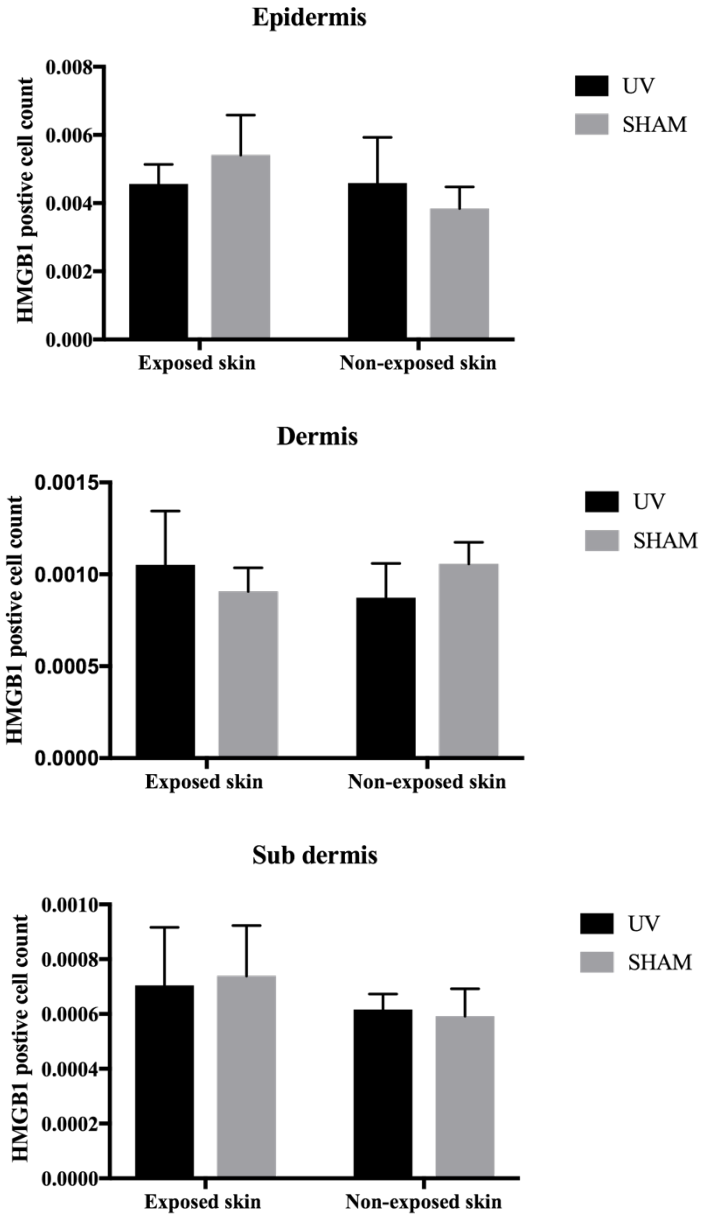


Figure B6. Cell counts of HMGB1 positively stained cells per μm^2 for the different layers of skin tissue. Skin samples were collected from the irradiated site and a proximal non-irradiated site, following exposure with either 0 (sham UVR) or 4 kj/m^2 of UVR (n=3-5 per group). The number of positively stained cells per mm^2 from epidermis, dermis and sub dermis were determined and used for a two-way ANOVA between the exposure groups and the irradiated and non-irradiated sites using GraphPad Prism 7 software (GraphPad Software Inc.; California, USA). However, no significant differences were observed between groups ($p>0.05$).

Outcome

1. Daily exposure of skin tissue to 4kj/m^2 of UVR for 7 days did not induce detectable changes in either H&E staining or HGMB1 immunohistochemistry between UV exposed and sham exposed group in any layer of skin tissue

References

Barker, D. J., Sanabria, F., Lasswell, A., Thrailkill, E. A., Pawlak, A. P. & Killeen, P. R. 2010. Brief light as a practical aversive stimulus for the albino rat. *Behav Brain Res,* 214**,** 402-408.

Catania, R. A., Schwacha, M. G., Cioffi, W. G., Bland, K. I. & Chaudry, I. H. 1999. Does uninjured skin release proinflammatory cytokines following trauma and hemorrhage? *Arch Surg,* 134**,** 368-373; discussion 373-364.

Peltz, E. D., Moore, E. E., Eckels, P. C., Damle, S. S., Tsuruta, Y., Johnson, J. L., Sauaia, A., Silliman, C. C., Banerjee, A. & Abraham, E. 2009. HMGB1 is markedly elevated within 6 hours of mechanical trauma in humans. *Shock,* 32**,** 17-22.

Team, R. C. 2016. R: A language and environment for statistical computing. *R Found Stat Comput.*

Appendix C: Source code written for operant conditioning boxes

Outlined below are the source codes developed for the behavioural testing within the operant conditioning boxes described in section 4.5.2. The experiment aimed to investigate if UVR administration had an effect on motivational drive determined with performance in the progressive ratio/concurrent choice behavioural paradigm described by Randell et al 2012. In this experiment the animal has the option to engage in lever pressing for a highly desirable sugar pellet or to eat the freely available standard rodent chow, after administration of either UVR or sham UVR.

Three separate codes were required to facilitate the appropriate training regime needed to train the animals to engage in lever pressing. The first phase of training, coded as *magazine training,* involved automatic delivery of a sugar pellet every 2 seconds, necessary to expose the animal to the chamber and delivery of sugar pellets. Next, animals progressed to the second phase, using the code *fixed ratio (FR) reinforcement schedule*. During this phase, animals are required to press a lever in order to trigger the release of a sugar pellets The FR schedule begins at 1 (1 lever press for 1 sugar pellet) but is gradually increased across training days to an FR5 schedule (5 lever presses for 1 sugar pellet). This was implemented by changing the "SET R = #" number, corresponding with the reinforcement counter, to the desired ratio at the beginning of each training day. Animals remain on an FR5 schedule until a steady output is reached. The final phase introduces the *progressive ratio reinforcement schedule* that will be used for the duration of the behavioural tests. During this phase there is an incremental raise in the number of lever presses required, every 15 reinforcements (FR1x15, FR2x15, FR3x15…).

Magazine training

```
\Magazine Training
\By Krystal Iacopetta
\March 03, 2017
\Automatic delivery of pellet every 2 seconds

\Input ; Output ---> Response

\this section is for outputs
^lever = 2          \left lever operate
^feeder= 3          \pellet dispenser operate
^house = 7          \house light
^light = 9          \cue light


S.S.1,     \Main Logical operator delivers pellet every 2 seconds

S1,
  #START: ---> S2

S2,
   0.1": ON 3, 9, 2; Z1  ---> S3

S3,
   6": OFF 3 ---> S2

S.S.2            \minute counter

S1,
 #START: SHOW 1, Minutes, X; SHOW 4, Seconds, S ---> S2

S2,
  1': ADD X; SHOW 1, Minutes, X ---> SX

S.S.3            \show pellets on screen

S1,
   #Z1: ADD B; SHOW 3, Pellets, B  ---> SX

S.S.4,             \Session Timer

S1,
   #START: SET M=30; ---> S2

S2,
 1": ADD S; Show 4, Seconds, S;
      IF S/60 >= M [@true, @false]
          @true: ---> S3
          @false: ---> SX
S3,
    2": OFF 3, 9, 2 ---> STOPKILL
```

Fixed ratio reinforcement schedule

```
\ Fixed Ratio Schedule
\ By Krystal Iacopetta
\ March 5 2017
\ FR 1 Schedule: This schedule can be increased by changing the
variable R


^leveron=2          \ out for lever
^lever=3            \ input for responding
^feeder=3           \ output for reinforcement
^light=9            \ light output for timeout indication



\ Variables

\ A = Total Lever Presses While Time-In
\ B = Total Pellets Received
\ C = Pellets Delivered in Grams
\ D = Highest Ratio Achieved
\ E = DIM of 5 Minute Response Bins
\ F = Time-Out Timer
\ G = 5 Minute Blocks of Responding
\ H = Time Out Clock
\ I = Ratio Increment
\ J = Time-Out Variable - 1 = timed out
\ K = Total Lever Presses While Time-Out
\ L = Time of Last Response
\ M =
\ N = Number of Reinforcements Before Ratio Increase
\ O =
\ P =
\ Q = Time to Complete Each Ratio
\ R = Reinforcement Ratio
\ S = Ratio Time Bin Incrementer
\ T = Ratio Time Timer
\ U = FR Counter - Resets after each reinforcement
\ V = Reinforcement Counter - Resets after N reinforcements
\ W =
\ X = Session Timer (in seconds)
\ Y = Session Duration
\ Z =

\ Z-Pulses:

\ Z1: off feeder, counts pellets, Time-Out Timer Reset
\ Z2: Ratio Incrementer
\ Z3: Time-Out Controller
\ Z4: Ratio Time Controller


DIM G = 6
```

```
DISK VARS = A, B, G, R


S.S.1,           \ Main Logical Operator

s1,

  #START: ON ^leveron, ^light; SET R = 5 ---> s2

s2,

  #Z3: ---> s1
  #R^lever: ---> s3

s3,

  0.04": ADD A; ADD U; SHOW 2, LEVER, A; Z2 ---> s4

s4,

  0.01": IF U >= R [@true, @false]
    @true: ON ^feeder; Z1; SET U = 0 ---> s2
    @false: ---> s2


S.S.2,           \ Feeder Control

s1,

   #START: ---> s2

s2,

  #Z3: ---> s1
  #Z1: ADD B; ADD V; SHOW 3, PELLETS, B; Z2 --->s3

s3,

  .1": OFF ^feeder ---> s2



S.S.3,           \ 5 Minute Blocks of Responding

s1,

   #R^lever: IF (X<=300) [@firsttrue, @firstfalse]
         @firsttrue: ADD G(0) ---> s1
         @firstfalse: IF (X>300) AND (X<=600) [@secondtrue,
@secondfalse]
                @secondtrue: ADD G(1) ---> s1
                @secondfalse: IF (X>600) AND (X<=900) [@thirdtrue,
@thirdfalse]
                      @thirdtrue: ADD G(2) ---> s1
```

```
                        @thirdfalse: IF (X>900) AND (X<=1200)
[@fourthtrue, @fourthfalse]
                                @fourthtrue: ADD G(3) ---> s1
                                @fourthfalse: IF (X>1200) AND
(X<=1500) [@fifthtrue, @fifthfalse]
                                        @fifthtrue: ADD G(4) ---> s1
                                        @fifthfalse: IF (X>1500)
[@sixthtrue, @sixthfalse]
                                                @sixthtrue: ADD G(5) --
-> s1
                                                @sixthfalse: ---> sx



S.S.8,              \ Session Timer  30 minutes

s1,

  #START ---> s2

s2,

  1": ADD X; SHOW 1, Timer, (X/60) ---> s3

s3,

  0.01":  IF X>=1800 [@END,@CONT]
                @END: ---> s4
                @CONT: ---> s2
s4,
3": OFF ^light, ^leveron ---> STOPABORTFLUSH
```

Progressive ratio reinforcement schedule

```
\ Progressive Ratio Schedule
\ By Krystal Iacopetta
\ March 5, 2017
\ Starts on FR1 and increments by 1 for every 15 reinforcements with
no limit
\ Coded with Time-Out Quit when no reinforcements for 2 minutes,
indicated by lights-out

^leveron=2          \ out for lever
^lever=3            \ input for responding
^feeder=3           \ ouput for reinforcement
^light=9            \ light output for timeout indication



\ Variables

\ A = Total Lever Presses While Time-In
\ B = Total Pellets Received
\ C = Pellets Delivered in Grams
\ D = Highest Ratio Achieved
\ E = DIM of 5 Minute Response Bins
\ F = Time-Out Timer
\ G = 5 Minute Blocks of Responding
\ H = Time Out Clock
\ I = Ratio Increment
\ J = Time-Out Variable - 1 = timed out
\ K = Total Lever Presses While Time-Out
\ L = Time of Last Response
\ M =
\ N = Number of Reinforcements Before Ratio Increase
\ O =
\ P =
\ Q = Time to Complete Each Ratio
\ R = Reinforcement Ratio
\ S = Ratio Time Bin Incrementer
\ T = Ratio Time Timer
\ U = FR Counter - Resets after each reinforcement
\ V = Reinforcement Counter - Resets after N reinforcements
\ W =
\ X = Session Timer (in seconds)
\ Y = Session Duration
\ Z =

\ Z-Pulses:

\ Z1: off feeder, counts pellets, Time-Out Timer Reset
\ Z2: Ratio Incrementer
\ Z3: Time-Out Controller
\ Z4: Ratio Time Controller


DIM G = 6
```

```
DIM Q = 50

DISK VARS = A, B, C, D, E, G, H, K, L, Q, R, S, T



S.S.1,           \ Main Logical Operator

s1,

  #START: ON ^leveron; SET I = 1; SET N = 15; SET R = 1---> s2

s2,

  #Z3: ---> s1
  #R^lever: ---> s3

s3,

  0.04": ADD A; ADD U; SHOW 2, LEVER, A; Z2 ---> s4

s4,

  0.01": IF U >= R [@true, @false]
    @true: ON ^feeder; Z1; SET U = 0 ---> s2
    @false: ---> s2



S.S.2,           \ Feeder Control

s1,

   #START: ---> s2

s2,

  #Z3: ---> s1
  #Z1: ADD B; ADD V; SHOW 3, PELLETS, B; Z2 --->s3

s3,

  .1": OFF ^feeder ---> s2



S.S.3,          \ Ratio Incrementer

s1,

  #Z2: ---> s2

s2,

  0.01": IF V>=N [@true, @false]
```

```
        @true: ---> s3
        @false: ---> s1

s3,

  0.01": SET R=(R+I); SET V=0; SHOW 4, RATIO, R; Z4 ---> s1



S.S.4,          \ Time-Out Timer Control

s1,

  #START: ON ^light --->s2

s2,

  #Z1: ---> s4
  1": IF R>2 [@true, @false]
        @true: ADD F; SHOW 5, T TIMER, F ---> s3
        @false: ---> s2
s3,

  0.01": IF F>=120 [@true, @false]
         @true: OFF ^light; ADD J; Z3 ---> sx
         @false: ---> s2

s4,

  0.01": SET F = 0 ---> s2



S.S.5,           \ 5 Minute Blocks of Responding

s1,

   #R^lever: IF (X<=300) [@firsttrue, @firstfalse]
         @firsttrue: ADD G(0) ---> s1
         @firstfalse: IF (X>300) AND (X<=600) [@secondtrue,
@secondfalse]
                 @secondtrue: ADD G(1) ---> s1
                 @secondfalse: IF (X>600) AND (X<=900) [@thirdtrue,
@thirdfalse]
                       @thirdtrue: ADD G(2) ---> s1
                       @thirdfalse: IF (X>900) AND (X<=1200)
[@fourthtrue, @fourthfalse]
                              @fourthtrue: ADD G(3) ---> s1
                              @fourthfalse: IF (X>1200) AND
(X<=1500) [@fifthtrue, @fifthfalse]
                                    @fifthtrue: ADD G(4) ---> s1
                                    @fifthfalse: IF (X>1500)
[@sixthtrue, @sixthfalse]
```

```
                                                    @sixthtrue: ADD G(5) --
-> s1
                                                    @sixthfalse: ---> sx



S.S.6,              \Time-Out Clock

s1,

  #START ---> s2

s2,

  #Z3: SHOW 6, TO, H ---> s1
   1": ADD H ---> s2



S.S.7,              \ Post Time Out Lever Presses

s1,

  #R^lever: ---> s2

s2,

  0.01": IF J>=1 [@true, @false]
           @true: ADD K ---> s1
           @false: ---> s1


S.S.8,              \ Time Since Last Response

s1,
   #START: ---> s2
s2,
   #R^lever: SET L = X; SHOW 5, LAST, L ---> s2


S.S.9,              \ Ratio Time Timer

s1,

    #START: ---> s2

s2,

    1": ADD T ---> s2



S.S.10,               \ Time To Complete Each Ratio
```

```
s1,
    #Z4: SET Q(S) = T; ADD S ---> s2

s2,
    0.1": SET T = 0 ---> s1

S.S.11,              \ Session Timer

s1,

  #START ---> s2

s2,

  1": ADD X; SHOW 1, Timer, (X/60) ---> s3

s3,

  0.01":  IF X>=1800 [@END,@CONT]
                @END: ---> s4
                @CONT: ---> s2

s4,
3": OFF ^light, ^leveron ---> STOPABORTFLUSH
```